

RESEARCH

L- and T-type calcium channels control aldosterone production from human adrenals

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Abstract

Aldosterone, which plays a key role in the regulation of blood pressure, is produced by zona glomerulosa (ZG) cells of the adrenal cortex. Exaggerated overproduction of aldosterone from ZG cells causes primary hyperaldosteronism. In ZG cells, calcium entry through voltage-gated calcium channels plays a central role in the regulation of aldosterone secretion. Previous studies in animal adrenals and human adrenal adrenocortical cell lines suggest that the T-type but not the L-type calcium channel activity drives aldosterone production. However, recent clinical studies show that somatic mutations in L-type calcium channels are the second most prevalent cause of aldosterone-producing adenoma. Our objective was to define the roles of T and L-type calcium channels in regulating aldosterone secretion from human adrenals. We find that human adrenal ZG cells mainly express T-type Ca_v3.2/3.3 and L-type Ca_v1.2/1.3 calcium channels. TTA-P2, a specific inhibitor of T-type calcium channel subtypes, reduced basal aldosterone secretion from acutely prepared slices of human adrenals. Surprisingly, nifedipine, the prototypic inhibitor of L-type calcium channels, also decreased basal aldosterone secretion, suggesting that L-type calcium channels are active under basal conditions. In addition, TTA-P2 or nifedipine also inhibited aldosterone secretion stimulated by angiotensin II- or elevations in extracellular K⁺. Remarkably, blockade of either L- or T-type calcium channels inhibits basal and stimulated aldosterone production to a similar extent. Low concentrations of TTA-P2 and nifedipine showed additive inhibitory effect on aldosterone secretion. We conclude that T- and L-type calcium channels play equally important roles in controlling aldosterone production from human adrenals.

Key Words

- ▶ T-type calcium channels
- ▶ L-type calcium channels
- ▶ aldosterone secretion
- ▶ primary aldosteronism

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Introduction

Aldosterone, a steroid hormone produced by zona glomerulosa (ZG) cells of the adrenal cortex, plays a key role in maintaining electrolyte and water balance, and thereby the control of blood pressure. Primary aldosteronism (PA),

caused by autonomous overproduction of aldosterone, is the most common form of secondary endocrine hypertension (Stowasser 2001). Approximately 10% of hypertensive patients have PA (Rossi *et al.* 2006,

Rossi 2011, Hannemann & Wallaschofski 2012), compared to 20% of individuals with resistant hypertension (Calhoun *et al.* 2002, Douma *et al.* 2008, Rossi 2011, Hannemann & Wallaschofski 2012). Idiopathic hyperaldosteronism and aldosterone-producing adenoma are the two most common subtypes of PA (Funder *et al.* 2008, Monticone *et al.* 2017). Notably, somatic mutations of potassium and calcium channels have been found in more than half of aldosterone-producing adenomas (Yang *et al.* 2018).

Intracellular Ca²⁺ regulates key steps in aldosterone biosynthesis from ZG cells (Barrett *et al.* 2016). Calcium controls the early rate-limiting step, the conversion of cholesterol to pregnenolone, by stimulating the transfer of cholesterol to CYP11A1 on the inner mitochondrial membrane (Cherradi *et al.* 1996). Calcium controls the late rate-limiting step, the conversion of deoxycorticosterone to aldosterone, by stimulating the production of NADPH, a required cofactor for CYP11B2 activity (Lifton *et al.* 1992, Rossier *et al.* 1996a, Lalevee *et al.* 2003, Wiederkehr *et al.* 2011). In ZG cells, a rise of intracellular Ca²⁺ can be generated either by releasing Ca²⁺ from intracellular stores or by increasing extracellular Ca²⁺ entry through voltage-gated calcium channels (Lotshaw 2001).

Extracellular Ca²⁺ entry through ZG voltage-gated calcium channels is necessary to maintain stimulated secretion induced by angiotensin II (Ang II) or K⁺, which are the two primary physiological aldosterone secretagogues *in vivo* (Barrett *et al.* 2016, Yang *et al.* 2018). ZG voltage-gated calcium channels are differentially expressed among mammalian species. Mainly two types of calcium currents have been identified in human, rat, mouse, and bovine ZG cells: high voltage-activated (L-type, including Ca_v1.1-1.4 subtypes) and low voltage-activated (T-type, including Ca_v3.1-3.3 subtypes) calcium channel currents (Cohen *et al.* 1988, Payet *et al.* 1994, Rossier *et al.* 1996b, Schrier *et al.* 2001, Hu *et al.* 2012). However, previous studies indicate that the activity of the T-type, but not that of the L-type channel, correlates with aldosterone secretion in dispersed preparations of animal ZG cells and human adrenocortical cell lines (Barrett *et al.* 1995, Rossier *et al.* 1996b, Imagawa *et al.* 2006, Akizuki *et al.* 2008). Thus, surprisingly recent clinical studies have found more than 25 different somatic Ca_v1.3 L-type calcium channels mutations in patients with PA (Yang *et al.* 2018), suggesting that in humans L-type calcium channels may also play an equally important role in controlling aldosterone production.

In this study, we assessed the contribution of T- and L-type calcium channels to the control of basal and stimulated aldosterone production from human adrenal

slices. We find that both channel types participate in the control of aldosterone production from human ZG cells that remained within adrenal tissue slices.

Materials and methods

Cell culture and chemicals

Human adrenocortical cell line, H295R (ATCC#: CRL-2128), was purchased from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 1% antibiotic solution, and 0.1% insulin, transferrin and selenium premix (BD Biosciences). Human embryonic kidney cell line, HEK293, was purchased from The Cell Bank of Chinese Academy of Science. Cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution. TTA-P2 was purchased from Alomone Laboratories; Angiotensin II and nifedipine were purchased from Sigma-Aldrich.

Aldosterone secretion of human adrenal gland

Adrenal gland samples were obtained from 28 patients (16 men and 12 women, age 36–70 years old) undergoing radical nephrectomy to remove kidney cancer and adjacent ipsilateral adrenal. The protocol for obtaining and using human adrenal tissues in this work was approved by the Ethics Committee of Shanghai Cancer Center (050434-4-1212B) and School of Life Sciences (2017-601), Fudan University. Written informed consent from all patients was obtained. All protocols were in accordance with institutional guidelines.

Human adrenal samples were prepared as previously described (Yang *et al.* 2016). Briefly, human adrenal samples were transported in cold PBS, and then kept in ice-cold bicarbonate-buffered saline containing: 140 mmol/L NaCl, 2 mmol/L KCl, 0.1 mmol/L CaCl₂, 5 mmol/L MgCl₂, 26 mmol/L NaHCO₃, and 10 mmol/L glucose, bubbled with 95% O₂ and 5% CO₂. Fat tissues around adrenal glands were removed. Tissues were embedded in 2.5% low-melting temperature agarose (2-hydroxyethylagarose, Sigma-Aldrich) and sectioned into 300 μm slices using DSK microslicer (DOSAKA). The slices were placed on Millicell-CM membranes (pore size 0.4 μm, Millipore) and incubated in Hanks MEM (Gibco) containing 1 mmol/L L-glutamine, 1 mmol/L CaCl₂, 2 mmol/L MgCl₂, 30 mmol/L HEPES,

12.8 mmol/L glucose, 5.24 mmol/L NaHCO₃, 12 mg/L bovine insulin, 0.12% ascorbic acid, and 20% horse serum (Gibco) at 37°C with 5% CO₂. Because the steroid synthesis profile of ZG cells may change during primary culture (Williams *et al.* 2014), in this study, we conducted relatively short time course secretion experiments. Slices were incubated for 2 h to determine basal production (control), and then treated with the indicated agents for 6 h to evaluate stimulated production. Slice culture supernatants were collected before and after treatment. Supernatant aldosterone concentrations were measured by an Aldosterone ELISA Kit (ENZO Life Science) as previously reported (Yang *et al.* 2016). For 62.5 pg/mL and 125 pg/mL aldosterone, the intra-assay co-efficients of variation were 5.2 and 6.2, and the inter-assay co-efficients of variation were 11.7 and 7.9. A standard curve (ranging from 3.9 pg/mL to 500 pg/mL) was conducted along with samples for every measurement. 10 µL supernatant (1:10 dilution in assay buffer) was used for aldosterone ELISA assay. In this condition, the mean percent recovery for 150 pg/mL and 50 pg/mL aldosterone were 93 and 98% respectively. Data were presented as fold over baseline to avoid ZG mass differences among the adrenal slices.

Immunofluorescence

Human adrenal glands were fixed in 4% paraformaldehyde at 4°C overnight, and transferred sequentially to 10, 20, and 30% sucrose/PBS. Tissues were embedded by OCT, and placed at -80°C. Tissues were sectioned (Leica CM1950 cryostat) into 20 µm slices and dried at room temperature. Slices were post-fixed in 4% paraformaldehyde for 15 min, washed (cold PBS), incubated in 0.5% PBST for 10 min, and blocked (10% horse serum, 0.3% PBST) for 2 h at room temperature. Slices were then incubated in primary antibody solution (primary antibody (mouse anti-Ca_v1.2: 1:200, AB11001554, Neuromab; mouse anti-Ca_v1.3: 1:200, AB10673964, Neuromab; mouse anti-Ca_v3.1: 1:200, AB2069421 Neuromab; rabbit anti-Ca_v3.2: 1:200, #ACC-025, Alomone; rabbit anti-Ca_v3.3: 1:200, #ACC-009, Alomone; rabbit anti-CYP11B2: 1:200, #ab168388, Abcam), 1% horse serum, and 0.3% PBST) for 1 day at 4°C. Slices were washed with PBS, and incubated in secondary antibody solution [(secondary antibody (Cy3-labeled goat anti-rabbit IgG or FITC-labeled goat anti-mouse IgG, 1:500, Beyotime), containing 1% horse serum, and 0.3% PBST) at 4°C overnight. After washing with PBS, slices were treated with DAPI. Confocal images were obtained using a Leica SP2 confocal microscope.

ZG cell isolation, RNA extraction and RT-PCR

The ZG layer was isolated from human adrenal glands using laser capture microdissection (Arcturus XT system, Life Technologies). Before laser capture microdissection, adrenal glands were incubated in RNAlater Solution (Life Technologies) at 4°C overnight, embedded with OCT, sectioned into 20 µm slices, and stained with HistoGene Staining Solution (Life Technologies). RNA was extracted (RNeasy Micro Kit, QIAGEN), reverse transcribed (SMART-Seq v4 Ultra Low Input RNA Kit, Clontech Laboratories). PCR was performed in 20 µL reactions containing: 2 µL of template, 0.4 µmol/L of each paired primer, and iQ SYBR Green Supermix (Bio-Rad). The thermocycling conditions were 95°C, 3 min; 40 cycles of 95°C, 15 s; 58°C, 30 s; 72°C, 30 s; and 72°C, 10 min. Primers used in PCR: Ca_v1.1: forward 5' CAGCAC TACAAC CAGTCG GA, reverse 5' CTCCAC CCAGGC AATACA GTC; Ca_v1.2: forward 5' CCTTTC TGGTTT AGCTGT GGG AAG ATCT, reverse 5' AATGCA AAGAGT TACTGAT TCCCGT TTCAG; Ca_v1.3: forward 5' GCACTA CGA GCA GTCCAA GA, reverse 5' GCTGCC GATTAC GATGAG GG; Ca_v1.4: forward 5' ACGGTG GAGATG CTTCTC AAATTG TACG, reverse 5' GGTGAC CTAAA GATCCT GAGGAG GC; Ca_v3.1: forward 5' GATCCT GACCCA GGAGGA CT, reverse 5' AAGAGC ACGTAG TTGCCGAA; Ca_v3.2: forward 5' CCTGGA GGAGAG CAACAAGG, reverse 5' GGTCTC CATCTC CACCTCCT; Ca_v3.3: forward 5' ACGCAT CTTTCT CACCGTGT, reverse 5' ATGGGC TTGAGG GAGGAGAT.

Cell transfection and electrophysiology

Plasmids for human Ca_v3.1, Ca_v3.2 and Ca_v3.3 channels (as previously reported (Cui *et al.* 2014)) were transiently transfected in H295R or HEK293 cells using jetPRIME (Polyplus) according to the manufacturer's instruction. For electrophysiological experiments, cells were used 24 h after transfection. Ca_v1.2 (Plasmid # 26572, Addgene) (Helton *et al.* 2005) or Ca_v1.3 (Plasmid # 26576, Addgene) (Xu & Lipscombe 2001) channels from Addgene (Cambridge, MA, USA) were transfected together with β₂ and α_{2δ}1 subunits (ratio 1:1:1) in H295R cells for 48–72 h before electrophysiological measurements. Whole-cell currents in the H295R cells were recorded using an Axopatch 200B amplifier (Axon Instruments). The bath solution contained (in mmol/L): 143 TEACl, 10 CaCl₂ (for Ca_v3 channel) or BaCl₂ (for Ca_v1 channel), 2 MgCl₂, 10 HEPES, and 10 Glucose, pH 7.4 (adjusted with TEOH). The internal solution contained (in mmol/L): 125 CsCl, 10 HEPES, 10 EGTA, 1 MgCl₂, 1 CaCl₂, 5 Mg-ATP, and

0.3 Tris-GTP (pH adjusted to 7.2 using CsOH). Recording pipettes (capillary tubing, BRAND) had resistances of 4–6 M Ω . All of the recordings were performed at room temperature. Currents were sampled at 10 kHz and filtered at 2 kHz, and corrected online for leak and residual capacitance transients using a P/4 protocol.

Data presentation and statistical analysis

Data analysis was performed with Clampfit 10.2 (Axon Instruments) and Origin 8.0 (OriginLab). The normal distribution of the data was checked by the Shapiro–Wilk test. Statistical analysis was performed using paired Student *t* tests unless indicated otherwise. The nonparametric Kruskal–Wallis test was used to compare groups that did not meet the assumption of normality. Values are given as means \pm s.e.m., *n* indicated the number of tested cells, patient samples or independent tests. *P* < 0.05 was considered statistically significant.

Results

L- and T-type calcium channels regulate basal aldosterone production from human adrenal slices

To determine which L-type calcium channel subtypes were expressed in human ZG cells, we isolated ZG cells by laser capture microdissection and tested for mRNA expression. As shown in Fig. 1A, mRNA for Ca_v1.2 and Ca_v1.3 but

not Ca_v1.1 and Ca_v1.4 was detected in ZG cells. All PCR products were confirmed by sequencing. To corroborate protein expression of Ca_v1.2 and Ca_v1.3 channels we used immunofluorescence. Immunodetection showed high expression of both Ca_v1.2 and Ca_v1.3 channel protein in the ZG layer of the human adrenal (Fig. 1B). By contrast, although the mRNA for all three T-type calcium channel subtypes was detected in ZG cells (Fig. 2A), the expression of T-type calcium channel protein, as reported by immunofluorescence, showed only strong expression for Ca_v3.2 and Ca_v3.3 channel protein. Signals for Ca_v3.1 channels were not detected (Fig. 2B, C and D) in the ZG layer of the human adrenal cortex, indicating a discrepancy between mRNA and protein expression. To assess the selectivity of these antibodies, and thus verify our putative expression findings, HEK293 cells were transfected with a vector encoding one of five channel subtypes to provide both positive and negative immunodetection controls. HEK293 cells expressing Ca_v3.1, Ca_v3.2, Ca_v3.3, Ca_v1.2 and Ca_v1.3 channels showed strong immunofluorescence signals only with antibodies against their own antigenic target; no signals were detected in either un-transfected HEK293 cells or in cells transfected with other channel family members (Supplementary Figs 1 and 2, see section on supplementary materials given at the end of this article). Thus, the normal human adrenal cortex, indeed expresses proteins for both Ca_v1.x and Ca_v3.x channels.

Next, we used the L-type calcium channel-specific inhibitor, nifedipine and the T-type calcium channel-specific inhibitor, TTA-P2, to test the contribution of each channel class to basal aldosterone secretion from human

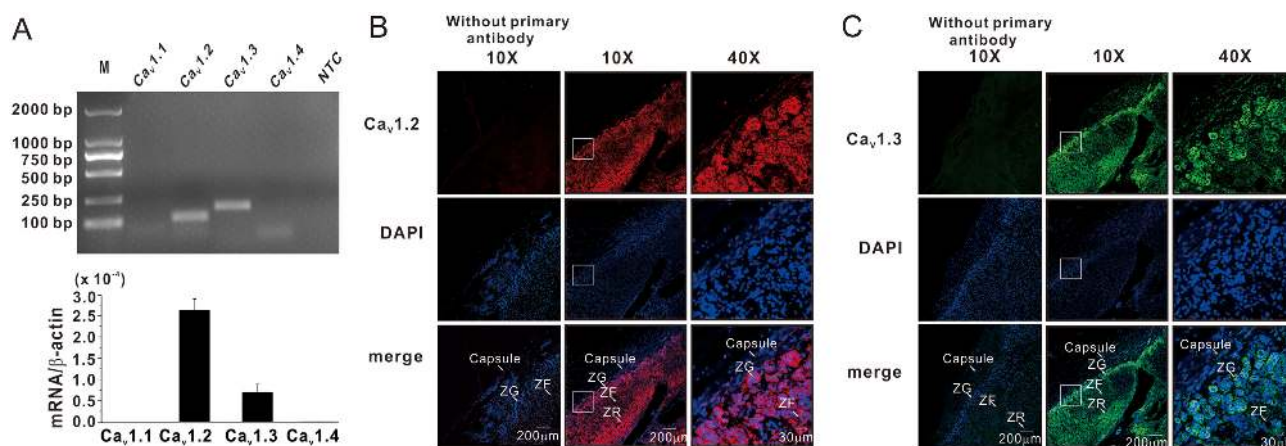
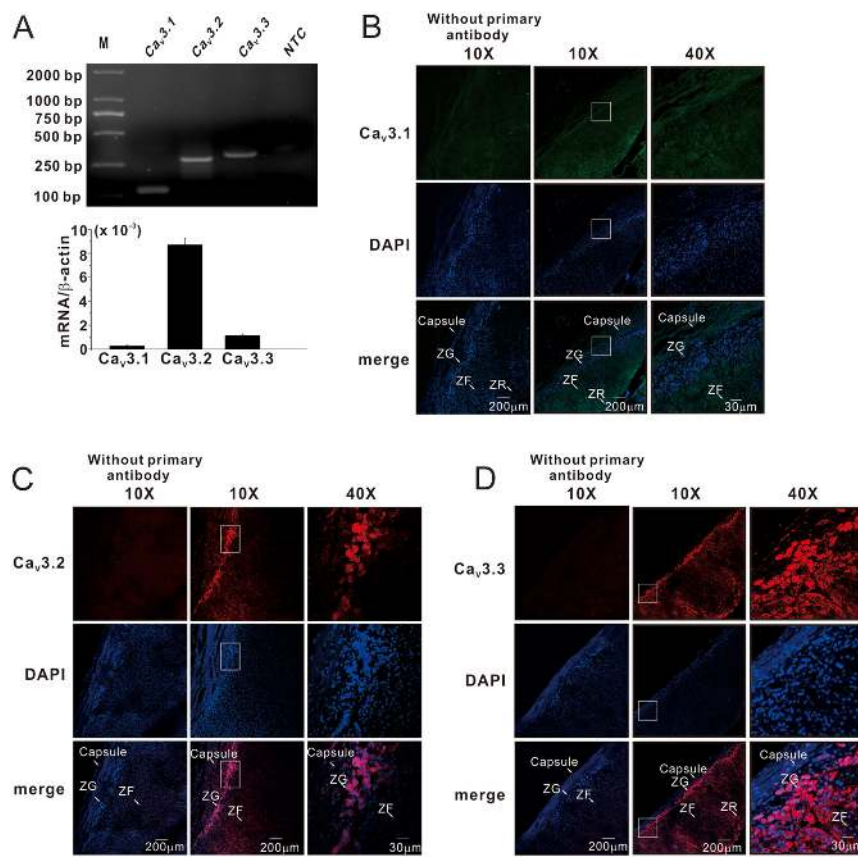


Figure 1

L-type calcium channel expression in the zona glomerulosa of human adrenals. (A) Human ZG cells express mRNA for L-type calcium channels. Zona glomerulosa was isolated using laser capture microdissection. Real-time PCR revealed message for L-type Ca²⁺ channels (Ca_v1.2 and Ca_v1.3, but not Ca_v1.1 and Ca_v1.4). (B and C) Representative examples of immunofluorescence images showing high expression of Ca_v1.2 and Ca_v1.3 channels in zona glomerulosa of the human adrenal cortex. Zona fasciculata (ZF), zona reticularis (ZR).

**Figure 2**

T-type calcium channel expression in the zona glomerulosa of human adrenals. (A) Human ZG cells express mRNA for T-type calcium channels. Zona glomerulosa was isolated using laser capture microdissection. Real-time PCR revealed message for T-type Ca²⁺ channels (Ca_v3.1, Ca_v3.2 and Ca_v3.3). (B, C and D) Representative examples of immunofluorescence images showing high expression of Ca_v3.2 and Ca_v3.3 but not Ca_v3.1 channels in zona glomerulosa of the human adrenal cortex.

adrenal slices. 500 nmol/L nifedipine significantly and consistently reduced aldosterone secretion from adrenal tissue slices prepared from ten individual patient adrenals (percent inhibition: $46 \pm 6\%$, $n=10$, $P<0.01$ compared with control by Student *t* tests, Fig. 3A). 2 μ mol/L TTA-P2 also significantly inhibited aldosterone secretion from adrenal tissue slices prepared from 11 individual patient adrenals (percent inhibition: $44 \pm 5\%$, $n=11$, $P<0.01$, Fig. 3B).

Although nifedipine is considered to be a highly selective L-type calcium channels blocker (Stengel *et al.* 1998), we tested if nifedipine could also partially inhibit T-type calcium channels at high nanomolar concentration. Because human adrenal ZG cells express both Ca_v3.2 and Ca_v3.3 channels, we evaluated the inhibition of Ca_v3.2 and Ca_v3.3 currents in H295R cells by 500 nmol/L nifedipine. Calcium currents were elicited by a 50-ms depolarizing pulse to -20 mV from a holding potential of -90 mV at 10 s intervals. 500 nmol/L nifedipine did not inhibit either Ca_v3.2 or Ca_v3.3 channel currents, whereas 2 μ mol/L TTA-P2 fully inhibited the currents (Fig. 4A and B, $n=5-6$). To confirm the efficacy of nitrendipine, we tested its effects on Ca_v1.2 and Ca_v1.3 channel currents at 500 nmol/L. As shown in Fig. 4C and D, 500 nmol/L nifedipine almost fully inhibited Ca_v1.2 and

Ca_v1.3 channel currents in H295R cells, in contrast to 2 μ mol/L TTA-P2 which had little effect ($n=5$). To rule out a nonselective inhibitory effect of these drugs at voltages other than -20 mV, we recorded the current-voltage relationship for each channel (Ca_v1.2, 1.3, 3.2, 3.3) and tested for drug-induced changes. At all voltages, 500 nmol/L nifedipine or 2 μ mol/L TTA-P2 remained selective for their channel targets (Supplementary Fig. 3). The data above indicate that the inhibition of L- or T-type calcium channel activity should account for the induced effects of 500 nmol/L nifedipine or 2 μ mol/L TTA-P2 reported here.

Effect of nifedipine and TTA-P2 on Ang II- and K⁺-induced aldosterone secretion from human adrenal slices

To assess the overall importance of L-type and T-type calcium channels in the production of aldosterone in human adrenals, we tested for nifedipine or TTA-P2 evoked modulation of Ang II- or K⁺-induced aldosterone secretion. As shown in Fig. 5A, 500 nmol/L nifedipine abrogated the stimulation of aldosterone production by 10 nmol/L Ang II (fold increase: Ang II, 2.4 ± 0.6 ; Ang II+nifedipine, 0.9 ± 0.1 , $n=6$, $P<0.05$ compared with

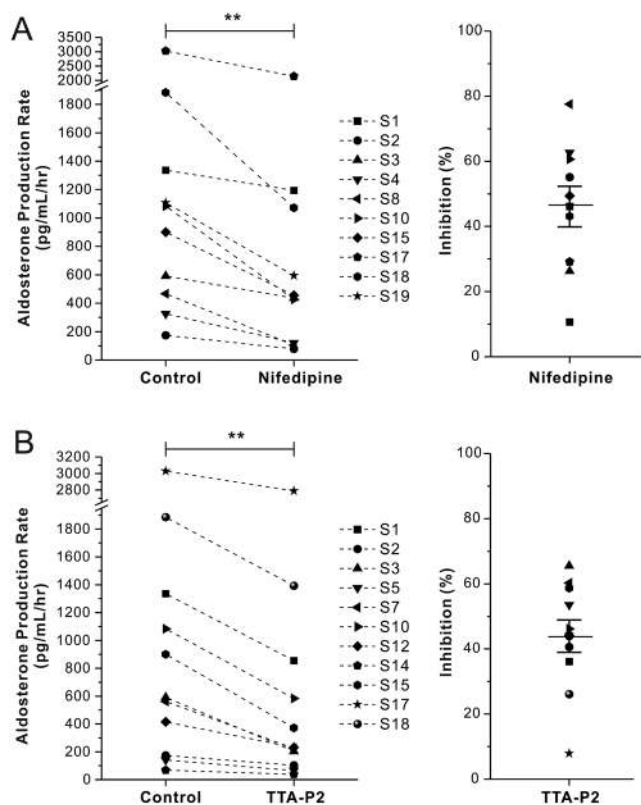


Figure 3

Effects of L- and T-type calcium channels on basal aldosterone secretion from human adrenal slices. (A) Specific L-type channel blocker, nifedipine (500 nmol/L), inhibits basal aldosterone production from human adrenal slices (left); Statistical analysis of nifedipine induced inhibition of aldosterone secretion from human adrenal slices (right). Data expressed as fold over control. $**P < 0.01$ compared with control ($n = 10$). (B) Specific T-type channel blocker TTA-P2 (2 $\mu\text{mol/L}$) reduced basal aldosterone production from human adrenal slices (left); Statistical analysis of TTA-P2-induced inhibition of aldosterone secretion from human adrenal slices (right). $**P < 0.01$ compared with control ($n = 11$). (S1–19 = samples from different individual patients.)

Ang II alone, Fig. 5B). 500 nmol/L nifedipine also abolished aldosterone production induced by High K⁺ (15 mmol/L) (fold increase: High K⁺, 2.4 ± 0.4 ; High K⁺+Nifedipine, 1.0 ± 0.2 , $n = 6$, $P < 0.05$ compared with High K⁺ alone, Fig. 5C and D). Interestingly, 2 $\mu\text{mol/L}$ TTA-P2 also abrogated the stimulation of aldosterone production by Ang II or High K⁺ from acutely prepared slices of human adrenals ($P < 0.05$, Fig. 6). Together, these data indicate that in human adrenals both T- and L-type calcium channel activity is required for stimulated aldosterone synthesis.

Additivity of effects of nifedipine and TTA-P2 on aldosterone production from human adrenal slices

Finally, we tested whether the combined application of nifedipine and TTA-P2 could reduce aldosterone secretion

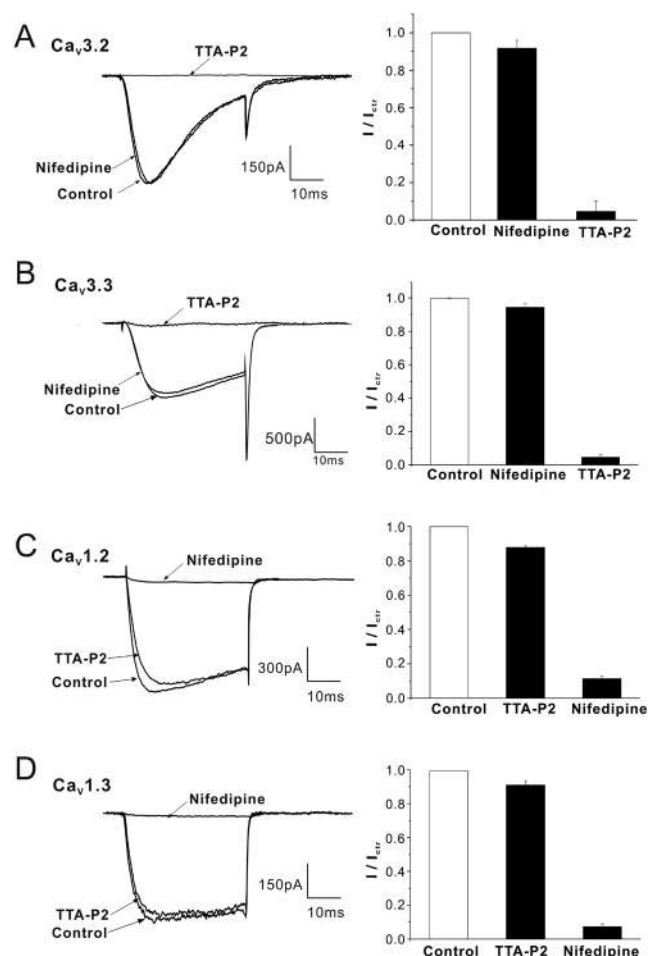
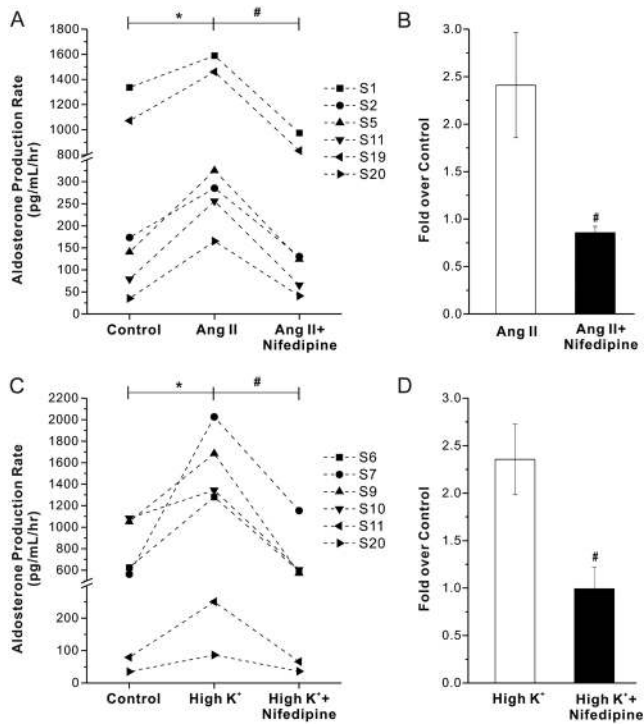


Figure 4

Effects of nifedipine (500 nmol/L) or TTA-P2 (2 $\mu\text{mol/L}$) on Ca_v3.2/3 and Ca_v1.2/3 channel currents in H295R cells. (A) Effects of L-type Ca²⁺ channel inhibitor nifedipine and T-type Ca²⁺ channel inhibitor TTA-P2 on Ca_v3.2 channel currents ($n = 6$). (B) Effects of 500 nmol/L nifedipine and 2 $\mu\text{mol/L}$ TTA-P2 on Ca_v3.3 channel currents ($n = 5$). (C) Effects of 500 nmol/L nifedipine and 2 $\mu\text{mol/L}$ TTA-P2 on Ca_v1.2 channel currents ($n = 5$). (D) Effects of 500 nmol/L nifedipine and 2 $\mu\text{mol/L}$ TTA-P2 on Ca_v1.3 channel currents ($n = 5$). Calcium currents were elicited by a 50-ms depolarizing pulse to -20 mV from a holding potential of -90 mV at 10 s intervals.

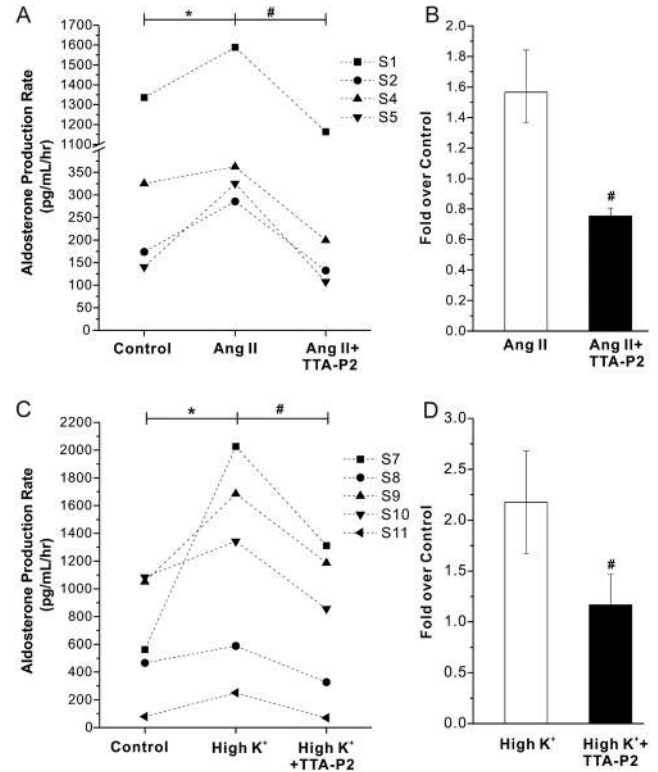
from human adrenal slices further than the level of inhibition induced by either drug alone. As shown in Fig. 7A, 2 $\mu\text{mol/L}$ TTA-P2 did not augment the degree of inhibition induced by nifedipine ($P > 0.05$ compared with nifedipine alone). Similarly, 500 nmol/L nifedipine did not increase the inhibition of basal aldosterone production from human adrenal slices produced by TTA-P2 ($n = 5$, $P > 0.05$ compared with TTA-P2 alone, Fig. 7B). To further test for drug additivity, we used lower concentrations of each antagonist, near the IC₅₀ for T or L channel current inhibition (Fig. 7C). As shown in Fig. 7D, the inhibition of basal aldosterone secretion from human adrenal slices by 200 nmol/L TTA-P2 was additive to that of 100 nmol/L

**Figure 5**

Effects of nifedipine on Angiotensin II (Ang II) - or high K⁺-stimulated aldosterone secretion from human adrenal slices. (A) Nifedipine abrogates Ang II-stimulated aldosterone secretion from human adrenal slices. (B) Statistical analysis of effect of nifedipine-induced inhibition of Ang II-stimulated aldosterone secretion. Data are presented as fold over control. * $P < 0.05$ compared with control ($n = 6$). # $P < 0.05$ compared with Ang II alone ($n = 6$). (C) Nifedipine abrogated high K⁺-stimulated aldosterone secretion from human adrenal slices. (D) Statistical analysis of nifedipine-induced inhibition of high K⁺-stimulated aldosterone secretion. Data are presented as fold over control. * $P < 0.05$ compared with control ($n = 6$). # $P < 0.05$ compared with high K⁺ alone ($n = 6$).

nifedipine ($n = 3$). Drug additivity was also demonstrable against agonist-stimulated secretion. The inhibition of Ang II (10 nmol/L) or high K⁺ (15 mM)-stimulated aldosterone secretion from human adrenal slices induced by either TTA-P2 (200 nmol/L) or nifedipine (100 nm/L) was less than that effected by these channel antagonists in combination (Fig. 8). These data indicate that both T- and L-type calcium channels maybe necessary to drive aldosterone production.

With aging, the cellular capacity to synthesize aldosterone declines (Hegstad *et al.* 1983, Nanba *et al.* 2018). Less aldosterone synthase (CYP11B2) is expressed in the human ZG and more is expressed in aldosterone-producing clusters (APCCs) found beneath the adrenal capsule (Nishimoto *et al.* 2010, 2016). APCCs, formed by non-neoplastic cells, increase with age and harbor mutations in the CACNA1D gene that encodes Ca_v1.3 channels (Nishimoto *et al.* 2015, Nanba *et al.* 2017).

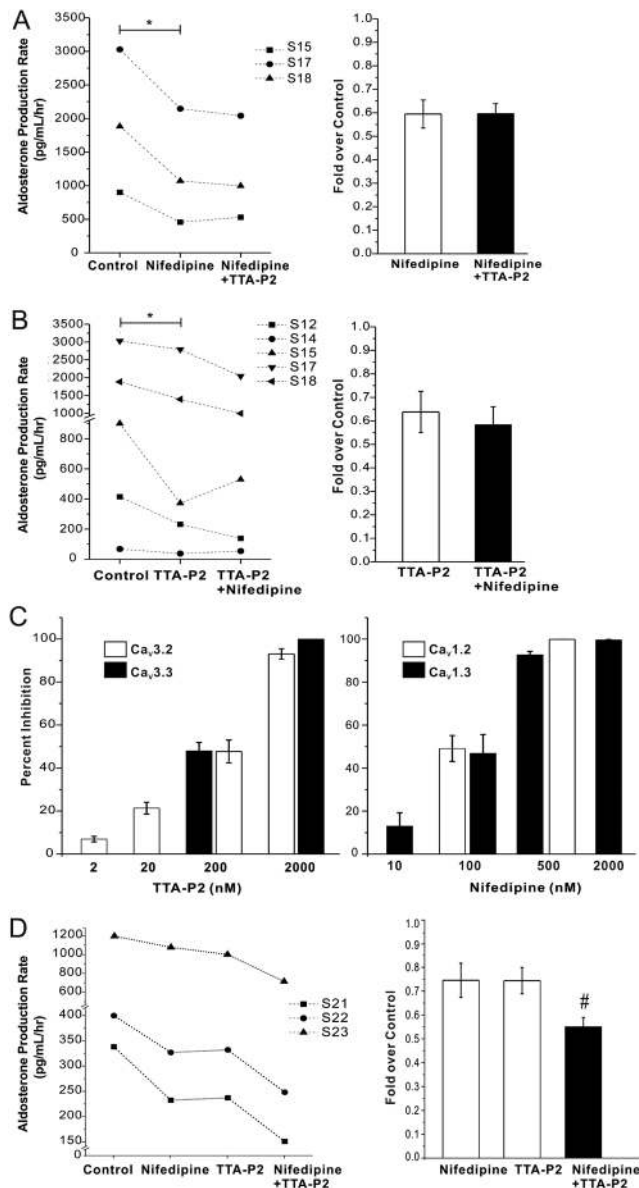
**Figure 6**

Effect of TTA-P2 on Angiotensin II (Ang II) - or high K⁺-stimulated aldosterone secretion from human adrenal slices. (A) TTA-P2 abrogates Ang II-stimulated aldosterone secretion from human adrenal slices. (B) Statistical analysis of TTA-P2 induced inhibition of Ang II-stimulated aldosterone secretion. Data are presented as fold over control. * $P < 0.05$ compared with control. # $P < 0.05$ compared with Ang II alone ($n = 4$). (C) TTA-P2 abrogates high K⁺-stimulated aldosterone secretion from human adrenal slices. (D) Statistical analysis of TTA-P2 induced inhibition of high K⁺-stimulated aldosterone secretion. * $P < 0.05$ compared with control. # $P < 0.05$ compared with high K⁺ alone ($n = 5$).

Therefore, we calculated the size effect (percent inhibition by nifedipine) as a function of patient age to determine if the nifedipine-induced inhibition of aldosterone production reported here increased with patient age. In the relatively small sample size of our study, we found no significant correlations with aging suggesting a role for normal human L-type calcium channels in the control of aldosterone synthesis (basal production: $R = -0.21$; $P = 0.56$ (Supplementary Fig. 4); Ang II stimulated: $R = 0.23$; $P = 0.66$ (data not shown); K-stimulated: $R = 0.24$; $P = 0.64$ (data not shown)).

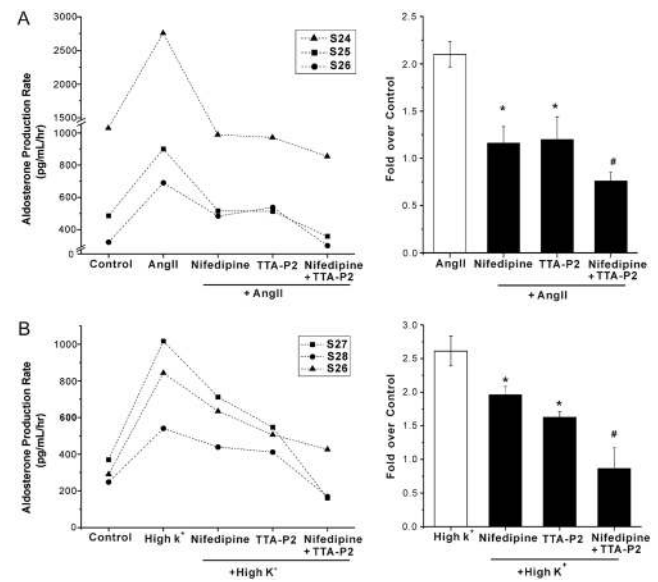
Discussion

Here, we report the novel findings that in human adrenal glomerulosa cells both basal and stimulated rates of aldosterone production depend on voltage-gated

**Figure 7**

Effect of combined exposure to nifedipine and TTA-P2 on the basal production of aldosterone from human adrenal slices. Both nifedipine and TTA-P2 maximally inhibit aldosterone secretion. (A) TTA-P2 does not alter nifedipine-induced inhibition of aldosterone production from human adrenal slices. (B) Nifedipine does not alter TTA-P2 inhibition of aldosterone production from human adrenal slices ($n = 5$). (C) Effects of different concentrations of nifedipine or TTA-P2 on Ca_v3.2/3 and Ca_v1.2/3 channel currents in H295R cells. (D) 100 nm/L nifedipine and 200 nm/L TTA-P2 showed additive effect on basal aldosterone secretion. # $P < 0.05$ compared with nifedipine or TTA-P2 alone.

calcium currents through T-type as well as L-type calcium channels. These findings are surprising because patch-clamp studies consistently report the basal membrane potential of ZG cells to be very hyperpolarized (around -80 mV), a potential at which high threshold L-type

**Figure 8**

Effect of combined exposure to nifedipine and TTA-P2 on Ang II or high K⁺-stimulated aldosterone secretion from human adrenal slices. (A) 100 nm/L nifedipine and 200 nm/L TTA-P2 showed additive inhibitory effect on Ang II-stimulated aldosterone secretion from human adrenal slices. # $P < 0.05$ compared with nifedipine or TTA-P2 alone. (B) 100 nm/L nifedipine and 200 nm/L TTA-P2 showed additive inhibitory effect on high K⁺-stimulated aldosterone secretion. # $P < 0.05$ compared with nifedipine or TTA-P2 alone.

calcium channels are not expected to be active. In contrast, low threshold T-type calcium channels are activated at more hyperpolarized potentials and have a permissive window of steady-state activity at more hyperpolarized potentials (Perez-Reyes 2003). When stimulated by low physiological concentrations of Ang II or small increases in extracellular potassium, the membrane potential of ZG cells can rapidly reach the permissive voltage window for persistent T-type, but not L-type, calcium channel activity, that allows steady-state calcium influx (Rossier 2016). Therefore, in primary cultured ZG cells (Python *et al.* 1993, Rossier *et al.* 1993, 1996b, 1998, Lotshaw 2001) or H295R cells (Akizuki *et al.* 2008), it is not surprising that Ang II- or K⁺-stimulated aldosterone secretion consistently has been reported to be efficiently inhibited by various T-type calcium channel blockers, and not inhibited by highly selective L-type calcium channel inhibitors such as nifedipine. Nevertheless, previously we reported that mouse ZG cells when retained within an adrenal slice spontaneously generate V_m oscillations (Hu *et al.* 2012), of high amplitude, which can reach depolarized membrane voltages close to but always below 0 mV, voltages at which L-type calcium channels are active. Therefore, the contribution of L-type calcium channels to the regulation

of aldosterone production previously may have been underestimated in studies of dispersed ZG cells that have lost the intrinsic ability to oscillate persistently.

Indeed, here we find that within human adrenal slices the L-type-specific blocker nifedipine significantly inhibited secretagogue-stimulated aldosterone secretion from ZG cells. Although some dihydropyridines that are considered to be specific L-type channel inhibitors, such as nicardipine and nifedipine also inhibit T-type calcium channels, nifedipine discriminates between T- and L-type calcium channel families (Stengel *et al.* 1998). Nifedipine selectively inhibits L-type calcium channels in human adrenocortical H295R cells (Rossier *et al.* 1996b, Imagawa *et al.* 2006, Akizuki *et al.* 2008, Yang *et al.* 2016) and in primary cultured human adrenal glomerulosa cells (Payet *et al.* 1994), and at 500 nM in our study had no effect on recombinant Ca_v3.2 and Ca_v3.3 channel currents expressed in human adrenal cells. Therefore, inhibition of L-type calcium channel activity should account for the nifedipine-induced reduction of aldosterone production reported here. Similar to previous studies (Dreyfus *et al.* 2010, Enyeart & Enyeart 2015), 2 μm TTA-P2 fully inhibits T-type calcium channels and has no effect on L-type calcium channels.

That nifedipine also inhibited aldosterone secretion from unstimulated human adrenal slices implies that L-type calcium channels are active even under basal condition. Again, we hypothesize that if human ZG cells within adrenal slices spontaneously generate V_m oscillations, as mouse ZG cells, this would enable L-type calcium channels to participate in regulating basal production. The direct proof of this prediction requires voltage recordings of human ZG cells by patch clamp. However, unfortunately we found that obtaining a gigaseal on adult human ZG cells (data not shown) was not possible. Rather, we used the very limited human adrenal samples for aldosterone secretion experiments. It is surprising that the degree of inhibition of aldosterone secretion evoked by nifedipine was equivalent to that of TTA-P2. At concentrations that produced maximal inhibition of L or T channel currents, no additivity of drug effects or synergy was observed, whereas at near half-maximal concentrations, nifedipine and TTA-P2 showed additive inhibitory effects on both basal and agonist-stimulated aldosterone secretion. Together, these observations suggest that in the human ZG layer, L- and T-type calcium channel flux may not be redundant but rather may each subserve a unique function that is required for the control of aldosterone production. Therefore, we propose a model for the regulation of aldosterone secretion from human

ZG cells by T- and L-type channels that can explain our findings. We predict that like mouse ZG cells, human ZG cells may also spontaneously oscillate, allowing the unique biophysical properties of each channel class to have distinct roles in controlling the oscillatory cycle. We hypothesize that by opening at more hyperpolarized potentials T-type channels may be principally responsible for recruiting L-type channels that, in turn, may provide the major calcium source of calcium required for the production of aldosterone; AngII or high K⁺ may increase oscillation frequency and thus control the magnitude of calcium entry and the production of aldosterone.

The samples used in this study were obtained from patients spanning a broad age range and would be expected to contain an age-dependent increase in APCCs. Indeed, with tissue remaining from some patient samples, we detected a very large CYP11B2 expressing APCCs in one of three samples (Supplementary Fig. 5). Approximately one-third of APCCs (Nishimoto *et al.* 2015) harbor mutations in Ca_v1.3 channels that lower the voltage dependence for channel activation or reduce channel inactivation (Azizan *et al.* 2013, Scholl *et al.* 2013), biophysical alterations that would make mutant channels more likely to participate in regulating calcium at hyperpolarized potentials. If mutant channels were the sole target of nifedipine action reported here, we would have expected an age-dependent increase in the efficacy of nifedipine, which we did not observe (Supplementary Fig. 4). Thus, we conclude that normal Ca_v1.3 channels as well as mutant Ca_v1.3 channels participated in the response to nifedipine. The relative contribution of each channel type to the inhibition of aldosterone synthesis induced by nifedipine in normal adrenal tissue is worthy of a future expanded study with a larger sample size.

In conclusion, our data demonstrate that L-type calcium channels play an important role in the regulation of aldosterone production from human ZG cells within a tissue context and that both T- and L-type calcium channels critically contribute to aldosterone production from human adrenals.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0259>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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