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Englerin A inhibits T-type CaV channels at low-micromolar concentrations

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Englerin A inhibits T-type channels

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List of abbreviations:

AngII, angiotensin II; AT1R, angiotensin II receptor type 1; BAPTA, 1,2-bis(o-
aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; CaV, voltage-gated calcium channel;
confidence interval, CI; DMSO, dimethyl sulfoxide; EA, englerin A; EC\textsubscript{50}, half-maximal excitatory concentration; FBS, fetal bovine serum; GFP, green fluorescent protein; GIRK, G protein-coupled, inward rectifying potassium channels; GnRH receptor, Gonadotropin releasing hormone receptor; GPCR, G protein-coupled receptor; HAC15, human aldosterone-producing zona glomerulosa cell line; HEK293 cells, human embryonic kidney cells; HVA, high-voltage-activated; IC\textsubscript{50}, half-maximal inhibitory concentration; IRES, internal ribosome entry side; K\textsubscript{2}P, two-pore potassium channels; LVA, low-voltage-activated; TASK, TWIK-related acid-sensitive potassium channels; TRPC, tetrameric transient receptor potential canonical ion channel; TWIK, tandem of P-domains in a weakly inward rectifying potassium channel
Abstract

Englerin A (EA) is a potent agonist of tetrameric transient receptor potential canonical (TRPC) ion channels containing TRPC4 and TRPC5 subunits. TRPC proteins form cation channels that are activated by plasma membrane receptors. They convert extracellular signals such as angiotensin II into cellular responses, whereupon Na\(^+\) and Ca\(^{2+}\) influx and depolarization of the plasma membrane occur. Via depolarization, voltage-gated Ca\(^{2+}\) (CaV) channels can be activated, further increasing Ca\(^{2+}\) influx. We investigated the extent to which EA also affects the functions of CaV channels using the high-voltage-activated L-type Ca\(^{2+}\) channel CaV1.2 and the low-voltage-activated T-type Ca\(^{2+}\) channels CaV3.1, CaV3.2 and CaV3.3. After expression of cDNAs in HEK293 cells, EA inhibited currents through all T-type channels at half-maximal inhibitory concentrations (IC\(_{50}\)) of 7.5 to 10.3 µM. In zona glomerulosa cells of the adrenal gland, angiotensin II-induced elevation of cytoplasmic Ca\(^{2+}\) concentration leads to aldosterone release. We identified transcripts of low- and high-voltage-activated CaV channels and of TRPC1 and TRPC5 in the human zona glomerulosa cell line HAC15. While no EA-induced TRPC activity was measurable, Ca\(^{2+}\) channel blockers distinguished T- and L-type Ca\(^{2+}\) currents. EA blocked 60% of the CaV current in HAC15 cells and T- and L-type channels analyzed at -30 mV and 10 mV were inhibited with IC\(_{50}\) values of 2.3 and 2.6 µM, respectively. While the T-type blocker Z944 reduced basal and angiotensin II-induced 24-hour aldosterone release, EA was not effective. In summary, we show here that EA blocks CaV1.2 and T-type CaV channels at low-micromolar concentrations.
Significance statement

In this study we showed that englerin A (EA), a potent agonist of TRPC4- or TRPC5-containing tetrameric TRPC channels and currently under investigation to treat certain types of cancer, also inhibits L-type voltage-gated CaV1.2 and T-type voltage-gated CaV3.1, CaV3.2 and CaV3.3 Ca\(^{2+}\) channels at low-micromolar concentrations.
**Introduction**

Englerin A (EA), a natural compound derived from the bark of the East African tree *Phyllanthus engleri*, is a potent agonist of TRPC channels containing TRPC4 and TRPC5 subunits (Akbulut et al., 2015). Even sub-micromolar concentrations of EA have a cytotoxic effect on renal cancer cells and other tumor cell lines (Akbulut et al., 2015; Caropreso et al., 2016; Carson et al., 2015; Ludlow et al., 2017; Muraki et al., 2017; Ratnayake et al., 2009; Sourbier et al., 2013; Sulzmaier et al., 2012; Williams et al., 2013). Mice injected intraperitoneally with EA show a reduction in locomotor activity from which they fully recover. TRPC4 and TRPC5 single knockout mice are partially protected from this EA-induced adverse effect, and TRPC4/TRPC5 double knockout mice are fully protected (Cheung et al., 2018). Binding studies showed that EA at micromolar concentrations partially displaced the dihydropyridine-type voltage-gated Ca$^{2+}$ (CaV) channel blocker nitrendipine from high-affinity binding sites in microsomal membrane fractions of rat brain cortex and that potassium-induced Ca$^{2+}$ influx in differentiated H9C2 cells was inhibited by 6 µM EA by up to 45% (Rodrigues et al., 2016). The differentiated H9C2 cells exhibit a cardiac phenotype, and an interaction between EA and L-type cardiac calcium channels, particularly CaV1.2, was assumed to occur. This assumption was supported by molecular modeling (Rodrigues et al., 2016) of EA into the KvAP- and KcsA-based models of the CaV1.2 pore domain (Tikhonov and Zhorov, 2009).

TRPC cation channels are activated by G protein-coupled receptors (GPCRs), e.g., in intestinal smooth muscle cells by muscarinic receptors (Tsvilovskyy et al., 2009), in pituitary neuroendocrine gonadotrophs by GnRH receptors (Beck et al., 2017). In these cells, they indirectly convert the extracellular signals acetylcholine and gonadotropin releasing hormone, respectively, into Ca$^{2+}$ influx and plasma membrane depolarization, thereby eliciting specific cellular responses. Ca$^{2+}$ influx is caused by the TRPC channels themselves, as they conduct
Ca$^{2+}$ as well as Na$^+$. At the same time, depolarization leads to the opening of CaV channels, resulting in additional Ca$^{2+}$ entry into the cell.

The family of voltage-gated Ca$^{2+}$ channels includes 10 members, which are divided into two subgroups, high-voltage-activated (HVA) channels and low-voltage-activated (LVA) or T-type channels. While the T-type channels consist only of the ion-conducting $\alpha_1$ pore subunits, the HVA channels contain additional subunits (Catterall, 2000; Catterall, 2011; Hofmann et al., 2014). HVA Ca$^{2+}$ channels are activated at membrane potentials of around -40 mV, whereas T-type channels open at more negative membrane potentials (Carbone and Lux, 1987) and are involved in spiking and oscillatory activities that play a role in the release of hormones and neurotransmitters and trigger the pacemaking activity of cells as well as cellular growth, differentiation, and proliferation (Carbone et al., 2014).

To investigate the direct effect of EA on the activity of CaV channels, we recorded currents from the HVA CaV1.2 channel and the LVA CaV3.1, CaV3.2 and CaV3.3 channels in the absence and presence of EA. At the same time, we studied the zona glomerulosa HAC15 cell line because in the zona glomerulosa cells of the adrenal cortex, angiotensin II (Ang II) leads to aldosterone release via its receptor, the angiotensin II receptor type 1 (AT1R), in which several ion channels are involved. Thus, inhibition of two-pore potassium (K2P) and G protein-coupled, inward rectifying potassium (GIRK) channels leads to depolarization, whereupon voltage-gated HVA and LVA Ca$^{2+}$ channels open (Enyeart and Enyeart, 2021). Gain-of-function mutations in the gene of ClCN-2 channels can also trigger depolarization and increased aldosterone release, which is associated with increased blood pressure (Fernandes-Rosa et al., 2018; Scholl et al., 2018). Gain-of-function mutations in the genes encoding the ion-conducting pores of CaV3.2 (Daniil et al., 2016; Scholl et al., 2015) and CaV1.3 channels (Azizan et al., 2013; Scholl et al., 2013) result in the same phenotype with increased aldosterone release and hypertension. Therefore, it could be that CaV3.2, a LVA channel, and CaV1.3, a HVA channel and closest relative of CaV1.2, predominate in
zona glomerulosa cells even under physiological conditions. The extent to which TRPC channels also play a role is not known, and we therefore examined zona glomerulosa HAC15 cells with respect to TRPC and CaV channels in the absence and presence of EA also with respect to Ang II-dependent aldosterone release.

Our results show that EA blocks HVA CaV1.2 and LVA CaV3.1, CaV3.2 and CaV3.3 at similar low-micromolar concentrations, that EA does not activate TRPC currents in zona glomerulosa cells of the adrenal cortex, and that, in contrast to the T-type channel blocker Z944, EA has no effect on Ang II-dependent aldosterone release.
Materials and Methods

Cell culture and transfection

The mouse HEK-TRPC4α cells (generously provided by Dr. M. X. Zhu, University of Texas, Houston, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM 41966-029, Gibco, Thermo Fisher Scientific, Waltham, USA), supplemented with 10% fetal bovine serum (FBS, Gibco), 100 µg/ml hygromycin B and 500 µg/ml G418 at 37°C and 5% CO2. The cells were transiently transfected with 1 µg of mouse TRPC1 cDNA encoding plasmid (pMax-TRPC1-IRES-GFP) using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, USA) and electrophysiological recordings were obtained from GFP-positive cells 48 h after transfection.

HEK293 cells stably expressing the cardiac ion-conducting CaV1.2 α subunit (HEK-CaV1.2; (Link et al., 2009)), cultured in Minimum Essential Medium (MEM 31095-029, Gibco), supplemented with 10% FBS and maintained at 37°C and 5% CO2, were transiently transfected with 1 µg of CaVβ2aN3 (β2) cDNA (pcAGGS-β2aN3-IRES-GFP, (Link et al., 2009)) using Fugene HD (Roche Applied Sciences) and electrophysiological recordings were obtained from GFP-positive cells 48 h after transfection.

The cDNA of human (h)CaV3.2 was subcloned into the pcDNA5/FRT vector to generate a Flp-In TRex 293 cell line, stably expressing the CaV3.2 cDNA in a tetracycline-inducible manner (Flp-In TRex 293 CaV3.2 cells; Flp-CaV3.2). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM 41965-039, Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco), 100 µg/ml hygromycin B and 2 mM Glutamax (Gibco) at 37°C and 5% CO2. Expression of the CaV3.2 cDNA was induced by tetracycline (1 µg/ml) 24 h before experiments.

HEK293 cells stably expressing T-type channels CaV3.1 (HEK-CaV3.1), CaV3.2 (HEK-CaV3.2) and CaV3.3 (HEK-CaV3.3) were generated and provided by Prof. Dr. Norbert
Klugbauer, Freiburg, and cultured at 37°C and 5% CO₂ in Minimum Essential Medium (MEM 31095-029, Gibco), supplemented with 10% FBS, 1% penicillin/streptomycin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and 500 µg/ml G418. CaV3.2 was cloned from human heart and CaV3.3 from human brain (Cribbs et al., 1998; Gomora et al., 2002). Plasmids were linearized and transfected into HEK293 cells, and G418 resistant HEK293 cells were isolated and screened for T-type currents. Generation of HEK293 cells stably expressing murine CaV3.1 followed the same procedure. Cloning of CaV3.1 from mouse brain is described in (Klugbauer et al., 1999).

HAC15 cells (ATCC CRL-3301), a clonal cell line from the NCI-H295R human adrenal gland epithelial carcinoma cell line (ATCC CRL-2128), were cultured at 37°C and 5% CO₂ in DMEM (21331-029, Gibco) supplemented with 10% HyClone Cosmic calf serum (Cytiva, Global Life Sciences Solutions USA LLC, Marlborough, USA), 1% Insulin-Transferrin-Selenium Premix (Corning Life Science, Corning, USA), 2.5 mM Glutamax, 15 mM HEPES and 1% penicillin/streptomycin.

**RT-PCR**

RNA was isolated and purified from HAC15 cells by using the RNeasy Plus Micro kit (Quiagen GmbH, Hilden, Germany) according to the manufacturer’s protocol and stored at -80°C until use. The isolated RNA was used for cDNA synthesis with the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). The synthesized cDNA was used as template to amplify CaV and TRPC transcripts in HAC15 cells by polymerase chain reactions. The PCR was performed with 1 µl phusion polymerase (NEB), 5 x HF buffer, 10 mM dNTPs, 10 pmol/µl of each primer (Table 1) in a total volume of 20 µl at 98°C for 30 s, followed by 35 cycles (10 s at 98°C, 10 s at 66°C, 15 s at 98°C) and 5 min at 72°C.
For RT-PCR controls RNA was isolated from HEK293 cells, cultured in MEM, supplemented with 10% FBS, and from NCI-H295R cells, cultured in DMEM/F-12 Medium. Both cell lines were maintained under standard cell culture conditions (37°C and 5% CO₂).

Cloning the full-length CaV3.2 cDNA from HAC15 cells

Primers for cloning the full-length CaV3.2 cDNA from HAC15 cells (see Figure 2A) are listed in Table 2. All fragments and the full-length cDNA were sequenced on both strands.

Western Blot

Western Blot (Figure 2B) was performed as described previously (Meissner et al., 2011). Proteins were denatured in SDS-denaturing buffer (4% SDS, 60 mM Tris, 0.005% bromophenol blue, 10% glycerol, 5% (v/v) 2-mercaptoethanol with pH 6.8) at 60°C for 20 min before applied to 8% SDS-PAGE NuPAGE Novex Bis-Tris gels (Invitrogen, life technologies, Carlsbad, USA). Afterwards, the protein was transferred onto a nitrocellulose membrane (Amersham Protran Premium 0.45 NC, GE Healthcare Life Sciences, Freiburg, Germany) and incubated at 4°C overnight with the mouse anti-MYC antibody (Roche, Basel, Switzerland). As loading control, the membrane was stripped and incubated at 4°C overnight with the rabbit anti-Calnexin antibody (ENZO Life Sciences, Farmingdale, NY, USA). Bound primary antibodies were visualized by the secondary, HRP-linked sheep anti-mouse antibody (NA9310) and donkey anti-rabbit antibody (NA9340, both GE Healthcare, Munich, Germany) and after addition of the Western lightning chemiluminescence reagent Plus (PerkinElmer, Waltham, USA), using the LAS-3000 analyzer (Fujifilm). Images were saved as TIFF files and transferred to CorelDRAW X7 (Corel Corporation, Ottawa, Canada) for clarity and labeling.

Aldosterone release
HAC15 cells were plated in 6-well plates at 800,000 cells per well using conditions and media as described above. After 24 hours, 6 or 12 µM englerin A, 1 or 10 µM Z944, 0.1% DMSO or just media (control) were added together with or without 10 nM angiotensin II (Ang II) to stimulate aldosterone release. Another 4 and 24 hours later, supernatants and trypsinized cells were separately collected. The aldosterone content of the supernatant was analyzed in the clinical laboratory of the Saarland University Medical Center, Homburg, using a chemiluminescence-immunoassay LIAISON aldosterone test (DiaSorin Inc., Stillwater, Minnesota, USA) and normalized to the protein content of the cell lysate from the respective well (pg aldosterone / µg protein). The latter was analyzed by a bicinchoninic acid (BCA) protein assay (Thermo Scientific). Probes for measuring aldosterone and protein content were blinded.

**Electrophysiology**

For patch clamp experiments, HEK-TRPC4α + TRPC1, HEK-CaV1.2 + β2, Flp-In TRex 293 CaV3.2 (Flp-CaV3.2), HEK-CaV3.1, HEK-CaV3.2, HEK-CaV3.3 and HAC15 cells were plated on small glass coverslips (1.2 cm ø, Thermo Fisher Scientific, USA). Whole-cell currents were recorded with an EPC9 amplifier (HEKA, Reutlingen, Germany) using the Patchmaster software (HEKA). Patch pipettes were pulled from borosilicate glass capillaries (Science Products GmbH, Hofheim, Germany) at a PC-10 puller (Narishige, Tokyo, Japan). To measure voltage-gated Ca²⁺ currents, the external solution contained (in mM) 102 NaCl, 10 CaCl₂, 5.4 CsCl, 1 MgCl₂, 20 TEA-Cl, 5 HEPES, 10 glucose (pH 7.4 with NaOH), and patch pipettes were filled with (in mM) 135 CsCl, 3 MgCl₂, 10 EGTA, 5 HEPES (pH to 7.4 with CsOH). Pipette tips were coated with SigmaCote (Sigma-Aldrich, Merck KGaA, Germany), and pipettes with resistances of 2-4 MΩ were used for measurements. After break-in 50 ms voltage ramps spanning from -100 mV to +100 mV were applied every 2 s from a holding potential (V_h) of -80 mV until Ca²⁺ inward currents reached a steady state. Then, cells
were clamped from -80 mV \( (V_h) \) to membrane potentials of -90 mV to +70 mV in 10 mV increments, for 400 ms each, with 2 s between the voltage steps. For TRPC current measurements in HEK-TRPC4α + TRPC1 and HAC15 cells external solution contained (in mM) 140 NaCl, 1 CaCl₂, 2.8 KCl, 2 MgCl₂, 10 HEPES, 10 glucose (pH 7.2), patch pipettes were filled with (in mM) 120 CsGlutamate, 1 MgCl₂, 8 NaCl, 10 CsBAPTA, 3.1 CaCl₂ (100 nM free Ca\(^{2+}\), calculated with webmaxc standard https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc/webmaxcS.htm), 10 HEPES (pH 7.2) and after break-in 400 ms voltage ramps spanning from -100 mV to +100 mV were applied every 2 s from a holding potential \( (V_h) \) of 0 mV. For CaV current measurements, ramp and step currents were corrected for linear leakage (patchmaster). All currents were filtered at 2.9 kHz and digitized at 400 μs intervals. The currents were normalized to the cell size (pA/pF) and the maximal current amplitude as well as the currents from steps to -30 mV, 0 mV or 10 mV were extracted and plotted versus voltage and time, respectively.

Drugs (Table 3) dissolved as stock in dimethylsulfoxid (DMSO, Sigma-Aldrich, Merck KGaA, Germany), were diluted in external solution and directly applied onto the patch clamped cell via an application pipette driven by air pressure (MPCU, Lorenz Meßgerätebau GmbH & Co KG, Katlenburg-Lindau, Germany).

**Statistical analysis**

RT-PCR results and Western Blots are representatives of at least three similar replicates. For patch clamp experiments the number in brackets \( (n) \) represents the number of measured single cells from two to four independent platings. If cells had been transfected, the data originate from two to three independent transfections. For detection of aldosterone release and protein content from HAC15 cell supernatant and pellet after treatment, probes, originating from six wells of two independent platings for each treatment, were blinded.
Patch clamp experiments were analyzed using Patch- and Fitmaster software (HEKA), IGOR Pro 6.31 (WaveMetrics), Microsoft Excel (Microsoft Corporation, Redmond, USA), OriginPro 8.6 (Microcal Software Inc., Northampton, USA) and Prism 9 (GraphPad Software, San Diego, USA). Half-maximal excitatory and inhibitory concentrations (EC$_{50}$, IC$_{50}$), Hill coefficients and their 95% confidence intervals (CI, asymmetrical, upper and lower limits) were calculated by sigmoidal fits of the dose response relations in IGOR Pro and GraphPad Prism. Final figures were prepared with CorelDRAW X7 (Corel Corporation, Canada). Statistical analysis was performed with Graph Pad Prism 9 (GraphPad Software). For the comparison of current inhibition at -30 mV and 10 mV (Figure 4I) a paired two-tailed Student’s t test was used and for more than two groups, comparing multiple treatments with a single control, a one-way ANOVA test with post-hoc Dunnett’s multiple comparison test (Figure 5). P values <0.05 were considered as statistically different. Values of current inhibition in the text are presented as mean ± standard deviation (SD), with the number (n) of experiments in brackets.
Results

Englerin A activates TRPC4/TRPC1 whole cell currents at sub-micromolar concentrations

At sub-micromolar concentrations englerin A (EA) activates TRPC4- and TRPC5-containing TRPC channels. Figure 1A shows EA-mediated TRPC4/TRPC1 whole cell currents at -80 mV (inward) and +80 mV (outward) plotted versus time. The whole cell currents revealed a soup ladle-shaped current-voltage-relationship (IV; Figure 1A, right panel), the typical signature of heteromeric TRPC4/TRPC1 currents (Muraki et al., 2017; Strubing et al., 2001). Whereas 1 nM EA had no effect on TRPC4α/TRPC1-expressing HEK293 cells, 3 nM EA already induced a detectable whole-cell current, which reached maximal amplitudes at about 1 μM EA. The dose-response relation revealed EC₅₀ values for TRPC4α/TRPC1 channel activation of 21.2 nM EA (95% confidence interval (CI): 11.8 to 49.6 nM), calculated from the outward current amplitudes, and 24.5 nM EA (95% CI: 21.0 to 29.0 nM), calculated from the inward current amplitudes (Figure 1B).

Englerin A inhibits heterologously expressed CaV1.2 and T-type CaV channels

While 30 nM EA was sufficient to induce a robust TRPC4/TRPC1 current, the same concentration had virtually no effect on the high-voltage-activated (HVA) dihydropyridine-sensitive CaV current in HEK293 cells expressing the cDNAs of the cardiac ion-conducting CaV1.2 α subunit and its auxiliary β2 subunit (Figure 1C, E). However, englerin A at a concentration of 6 μM inhibited the CaV1.2 currents by 50.6 ± 7.1% (n=5; Figure 1D, E).

To study the effect of englerin A on low-voltage-activated (LVA) T-type Ca²⁺ channels, we first cloned the cDNA of CaV3.2 from the human adrenal carcinoma HAC15 cells (Figure 2A) and generated a Flp-In TRex 293 cell line, which inducibly expresses the CaV3.2 cDNA under the control of tetracycline (Flp-CaV3.2). The CaV3.2 protein was detectable by its MYC-tag in cell lysate after 24 hours tetracycline induction (Figure 2B). Voltage-gated Ca²⁺
currents with typical current-voltage relationship of T-type CaV channels were recorded with maximal current amplitude at -30 mV (Figure 2C). Non-induced cells revealed just a tiny voltage-gated Ca\(^{2+}\) current with maximum amplitude at about 0 mV (Figure 2D, blue trace), which might represent endogenously expressed voltage-gated Ca\(^{2+}\) channels in Flp-In TRex 293 cells. Englerin A at 6 µM reduced the CaV3.2 current in Flp-CaV3.2 cells by 26.0 ± 11.4% (n=13), at 30 µM by 91.3 ± 4.9% (n=8; Figure 2D, blue and red traces). The dose-response relation revealed a half-maximal englerin A concentration for current inhibition (IC\(_{50}\)) of 10.3 µM (95% CI: 6.7 to 14.9 µM; Figure 2E). Experiments on HEK293 cells stably expressing CaV3.2 (HEK-CaV3.2) revealed a similar IC\(_{50}\) of 8.0 µM (95% CI: 7.1 to 9.0 µM) englerin A (Figure 2F) and confirmed the results from tetracycline-inducible CaV3.2 expressing cells. Next, we tested whether englerin A also affects CaV3.1 and CaV3.3 currents in HEK293 cells stably expressing the respective T-type channel. Our experiments revealed half-maximal englerin A concentrations of 7.5 µM (95% CI: 5.9 to 10.8 µM) and 9.9 µM (95% CI: 8.7 to 11.3 µM) for CaV3.1 (Figure 2G) and CaV3.3 (Figure 2H). Thus, englerin A inhibits all LVA T-type CaV channels with a similar potency and does not discriminate between the subtypes. As pharmacological control, we used the non-specific T-type inhibitor Z944 (Tringham et al., 2012), which, at 1 µM, inhibited about 90% of the CaV current in the CaV3.1, CaV3.2 and CaV3.3 expressing HEK293 cells (Figure S2A).

Expression and pharmacological characterization of CaV channels in the aldosterone-producing zona glomerulosa HAC15 cells

Beside CaV3.2, HAC15 cells endogenously express transcripts of additional voltage-gated Ca\(^{2+}\) channel α subunits including the ion-conducting pores of CaV1.2, CaV1.3, CaV1.4, CaV2.2, CaV3.1 and CaV3.3 (Figure 3A, left). They also express transcripts of auxiliary CaVβ and α2δ subunits, except of α2δ-4 (Figure 3A, right). Since englerin A is a potent activator of TRPC4 and TRPC5 (Akbulut et al., 2015), we analyzed HAC15 cells for their
TRPC expression. While TRPC1, TRPC5 and TRPC6 transcripts were detected by RT-PCR (Figure 3B), no englerin A-mediated current was observable in HAC15 cells (Figure 3C). However, whole-cell patch clamp experiments revealed prominent voltage-gated Ca\(^{2+}\) currents in HAC15 cells, with a maximal current amplitude at voltage steps to 0 mV (Figure 4A). To dissect the LVA T-type and HVA L-type CaV currents, CaV channel blockers were applied and the current amplitudes at -30 mV (LVA) and 10 mV (HVA) were analyzed (Figure 4B-I). 10 µM mibefradil (Ro 405967), which blocks HVA and LVA CaV channels (Lacinova et al., 1995; Viana et al., 1997), reduced the current amplitude by 75.2 ± 8.3% and 70.2 ± 9.3% at -30 mV and at 10 mV (n=8; Figure 4B, I). The T-type CaV channel blocker Z944 (Tringham et al., 2012) at 10 µM inhibited 74.5 ± 7.4% and 38.5 ± 8.9% of the CaV current at -30 mV and 10 mV (n=8; Figure 4C, I), indicating, that the current in the presence of Z944 mainly represents HVA CaV currents (Figure 4C, red trace). This fraction of the current was further reduced by subsequent addition of the L-type blocker verapamil (Figure S4G). At 1 µM, Z944 inhibited 57.2 ± 9.2% and 41.7 ± 15.9% of the CaV current at -30 and 10 mV (n=9; Figure S2B). With an inhibition of 18.5 ± 8.7% at -30 mV and 34.7 ± 8.2% at 10 mV, Ethosuximide at 10 mM had only minor effects on the CaV current in HAC15 cells (n=7; Figure 4D, I). SKF 96365 at 10 µM reduced the CaV current by 52.6 ± 9.0% at 10 mV and by 31.5 ± 12.7% at -30 mV (n=9; Figure 4E, I). The L-type CaV channel blocker verapamil inhibited 58.5 ± 14.4% of the CaV current at 10 mV and 33.3 ± 16.4% at -30 mV (n=8; Figure 4F, I). The remaining currents (Figure 4F, red trace) were further blocked by addition of Z944 (Figure 4F, blue trace), resulting in a final reduction by 68.4 ± 27.2% at -30 mV and by 79.6 ± 12.5% at 10 mV (Figure 4F, I). Like CaV1.2 and CaV3 channels in HEK293 and Flp-In TRex 293 cells, englerin A inhibited endogenous voltage-gated Ca\(^{2+}\) currents in HAC15 cells. 6 µM englerin A blocked the CaV current by 63.6 ± 8.9% at -30 mV and by 50.1 ± 15.2% at 10 mV (n=8; Figure 4G-I). The dose-response relation revealed very similar IC\(_{50}\) values at -30 mV (2.3 µM EA, 95% CI: 1.2 to 4.5 µM), 0 mV (2.4 µM EA, 95% CI: 1.1 to 5.1 µM) and 10 mV
(2.6 µM EA, 95% CI: 1.3 to 4.6 µM; Figure 4H). While ethosuximide, SKF 96365 and verapamil preferentially inhibited the HVA fraction of the CaV current in HAC15 cells at 10 mV, Z944 and englerin A preferentially blocked the LVA fraction at -30 mV (Figure 4I, see ratio of inhibition at -30 mV and inhibition at 10 mV, calculated from the single experiments). Figure S4 shows the currents at voltage steps to -30 mV (left panels) and 10 mV (right panels) before and in the presence of all used Ca²⁺ channel blockers.

Z944 inhibits aldosterone release in HAC15 cells

T- and L-type channels, are involved in angiotensin II-mediated release of aldosterone from aldosterone-producing zona glomerulosa cells (Chen et al., 1999; Spat and Hunyady, 2004). Under control conditions (control or control + DMSO) we could detect a basal release of 3 to 4 pg aldosterone per µg protein (from initially 800.000 plated cells) within 4 hours (Figure 5A) and about 11 pg aldosterone per µg protein within 24 hours (Figure 5C). This basal release was not different in the presence of 12 µM englerin A or 1 µM Z944 after 4 hours and 6 µM englerin A after 24 hours, but significantly (p = 0.0009) reduced in the presence of 10 µM Z944 after 24 hours. Addition of angiotensin II (Ang II) at 10 nM increased the amount of aldosterone in the supernatant about 2-fold within 4 hours (Figure 5B) and about 6-fold within 24 hours (Figure 5D). Neither 12 µM englerin A or 1 µM Z944 affected the 4-hour nor 6 µM englerin A the 24-hour angiotensin-mediated aldosterone release (Figure 5B, D) but, the T-type channel blocker Z944 (10 µM) reduced the Ang II-dependent aldosterone release by almost 50% within 24 hours of incubation.
Discussion

In the present work, we investigated the extent to which englerin A, a potent agonist of TRPC4- and TRPC5-containing TRPC channels, affects the activity of voltage-gated Ca\(^{2+}\) channels. We performed whole-cell patch clamp experiments on CaV1.2, CaV3.1, CaV3.2 and CaV3.3 channels, heterologously expressed in HEK293 and Flp-In TReX 293 cells and pharmacologically dissected endogenous low- and high-voltage-activated Ca\(^{2+}\) currents in the aldosterone-producing adrenal zona glomerulosa cell line HAC15.

While EA activates outward and inward currents through TRPC4/TRPC1 channels with EC\(_{50}\) values of 20.1 and 23.8 nM, respectively, at 30 nM it had no effect on CaV1.2 currents, and at 6 µM it inhibited these currents by about 50\% (Figure 1). In addition, englerin A resulted in half-maximal inhibitory concentrations of 7.7-10.3 µM for all LVA channels, CaV3.1, CaV3.2 and CaV3.3, expressed in HEK293 cells, without discriminating between the subtypes (Figure 2). Our results show that CaV1.2 and all T-type CaV currents are inhibited by englerin A at similar low-micromolar concentrations.

The zona glomerulosa cells of the adrenal gland release aldosterone in an Ang II-dependent manner and two distinct voltage-gated Ca\(^{2+}\) channel currents, L- and T-type, had been identified in adrenal glomerulosa cells being important for aldosterone release (Matsunaga et al., 1987; Uebele et al., 2004). Gain-of-function mutations in the genes of the ion-conducting α1 pores of CaV1.3 and CaV3.2 lead to increased release of aldosterone in humans, which is associated with altered extracellular potassium concentration and hypertension, among other effects (Azizan et al., 2013; Daniil et al., 2016; Fernandes-Rosa et al., 2014; Ortner et al., 2020; Reimer et al., 2016; Scholl et al., 2013; Scholl et al., 2015). Therefore, to further characterize the ability of englerin A to influence CaV currents, we examined the zona glomerulosa HAC15 cell line. These cells express transcripts of the ion-conducting pores of CaV1.2, CaV1.3, and all three subtypes of LVA channels (CaV3.1,
CaV3.2 and CaV3.3), among others (Figure 3A). In these cells, endogenously present CaV channels are also inhibited by a maximum of 60% at englerin A concentrations of 6 µM.

Due to the expression of several potassium leak channels such as TASK-1, TASK-2, TASK-3, the TWIK-related K⁺ channel 1 and GIRK, under non-stimulated conditions, the membrane potential of zona glomerulosa cells closely follows the potassium resting potential at about -80 mV (Seccia et al., 2018; Spat and Hunyady, 2004). An increase in extracellular K⁺ concentration or inhibition of K⁺ channels by Ang II via its receptor (AT1R) leads to depolarization of the cell membrane, opening of voltage-gated Ca²⁺ channels, and an increase in intracellular Ca²⁺ concentration, the main trigger for aldosterone biosynthesis (Bollag, 2014; Spat and Hunyady, 2004). Binding of Ang II to AT1R also leads to Gαq-mediated signaling and IP₃-mediated release of Ca²⁺ from the endoplasmic reticulum. Aldosterone is not released exocytotically but as a steroid can freely permeate the plasma membrane, i.e., its increased synthesis leads to increased release.

Englerin A (6 or 12 µM) had no effect on the basal and Ang II-dependent release of aldosterone from HAC15 cells (Figure 5). In contrast, the T-type Ca²⁺ channel blocker Z944 (10 µM) reduced basal and Ang II-dependent 24-hour aldosterone release by almost 50%. Z944 selectively inhibits T-type channels with half-maximal inhibitory concentrations of 50 to 160 nM by binding in the pore region of inactivated CaV3 channels (Tringham et al., 2012; Zhao et al., 2019). CaV3 channels are activated at low membrane potentials - even below the resting potential - and hyperpolarization is often required for de-inactivation. In contrast, nothing is yet known about the mechanisms by which englerin A inhibits LVA CaV (and CaV1.2) currents. Three amino acid residues in the lower gate of TRPC5 have been suggested as binding site for englerin A (Jeong et al., 2019), and binding studies showed that EA partially displaced the dihydropyridine-type CaV blocker nitrendipine from high-affinity binding sites (Rodrigues et al., 2016). That englerin A does not inhibit aldosterone release from HAC15 cells may be due to the fact, that it is not stable in the 24-hour aldosterone
release assay we employed and is converted to inactive products. Englerin A has been shown to be stable for at least 4.5 hours in canine and human plasma, but less time in rat and mouse plasma (Carson et al., 2015). The low stability of EA in rodent serum may account for the fact that the adverse effects that occurred in englerin A-treated mice were transient (Cheung et al., 2018). However, englerin A, even at 12 µM, did not affect 4-hour aldosterone release either, and our control experiments revealed, that englerin A, prepared in external saline at a concentration of 12 µM and kept 24 hours at room temperature, did still inhibit more than 80% of the CaV current in CaV3.2 expressing cells (Figure S2C).

In summary, our results show that englerin A, which potently activates TRPC4- and TRPC5-containing TRPC channels at sub-micromolar concentrations, blocks the voltage-gated CaV1.2 and the T-type CaV3.1, CaV3.2 and CaV3.3 channels at low-micromolar concentrations.
Acknowledgements

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Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.
Authorship contributions

Participated in research design: Flockerzi, Beck

Conducted experiments: Wardas, Beck

Contributed new reagents or analytic tools: Schneider, Klugbauer, Flockerzi

Performed data analysis: Wardas, Beck

Wrote or contributed to the writing of the manuscript: Wardas, Flockerzi, Beck
References


Footnotes

Financial support

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Person to receive reprint requests

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Financial disclosure statement

No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

Figure 1 – Effect of englerin A on TRPC4/TRPC1 channels and CaV1.2 currents. (A) Whole cell currents recorded from TRPC4α and TRPC1 co-expressing HEK293 cells (HEK-TRPC4α + TRPC1), extracted at +80 mV and -80 mV during 400 ms voltage ramps spanning from -100 mV to 100 mV, applied every 2 s from a holding potential of 0 mV, plotted versus time. The bar indicates application of increasing concentrations (0.1 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM and 1 µM) of englerin A (EA). Basic currents before EA application had been subtracted. Current-voltage relationships of the net EA-mediated currents are shown on the right panel. (B) Maximal amplitudes of the net EA-induced in- and outward currents at -80 mV and +80 mV plotted versus the EA concentration. The dose response relations (sigmoidal fits, black traces) revealed EA concentrations for half-maximal activation (EC_{50}) of 24.5 nM (95% confidence interval (CI): 21.0 to 29.0 nM) and 21.2 nM (95% CI: 11.8 to 49.6 nM) with a Hill coefficient (cooperativity) of 1.2 (95% CI: 1.0 to 1.3) and 1.0 (95% CI: 0.6 to 1.6) for in- and outward currents, respectively. (C, D) Voltage-gated inward currents detected from CaV1.2 expressing HEK293 cells (HEK-CaV1.2) co-expressing CaVβ2aN3 (β2) before (ctr, black) and in the presence (red) of 30 nM (C) and 6 µM EA (D). Currents were induced by 50 ms voltage ramps spanning from -100 mV to 100 mV (V_h -80 mV). (E) EA-dependent inhibition (in percent) of CaV1.2 currents from experiments as in C and D. Data represent means ± SEM (A left, B), ± SD (E), means (A right) and example traces (C, D). Circles in E represent single data points. Numbers of measured cells are indicated in brackets.

Figure 2 – Functional expression of human CaV3.2. (A) Scheme of the 2353 amino acid (aa) human (h)CaV3.2 protein (top) with transmembrane segments S1 to S6 of domains I, II, III and IV as black bars. The 7062 bp hCaV3.2 full-length cDNA was obtained by subcloning overlapping RT-PCR fragments 1-5 corresponding to transcript 1 of CACNA1H in the
Ensembl Genome Browser (gene accession number NM_021098.2), and subcloned into pcDNA5/FRT/TO. Fragments 4a, 5a, 5b and 8 contained deletions and were not used. Positions of forward (f) and reverse primers (r) are indicated. The respective primers are shown in Table 2. (B) Western blot of protein lysates from Flp-In TRex 293 cells inducibly expressing the cDNA of hCaV3.2, 3’-tagged with the polypeptide EQKLISEEDL (MYC), with (Tet +) and without (Tet -) 24 hours induction by 100 ng/ml tetracycline, using an anti-MYC antibody (left). The membrane was stripped and reprobed with an anti-Calnexin (CNX) antibody as loading control (right). The original Western Blot is shown in Figure S1. (C) Inward currents detected from tetracycline-induced (24 hours) cells during a 400 ms depolarization step to -30 mV from a holding potential (V_h) of -80 mV. The current-voltage relationship (insert) was obtained by voltage steps from -90 mV to +70 mV (10 mV increments; V_h -80 mV). (D) CaV3.2 currents before (ctr, black) and during application of 6 µM (blue), 12 µM (orange) and 30 µM (red) englerin A 24 hours after tetracycline induction. Currents of non-induced cells are shown in dark blue. (E) The dose-response relation revealed an IC_{50} of 10.3 µM (95% confidence interval (CI): 6.7 to 14.9 µM) englerin A at a Hill coefficient of 2.0 (95% CI: 0.9 to 4.8) for CaV3.2 inhibition. (F-H) Dose-dependent inhibition of englerin A on T-type currents in HEK293 cells stably expressing CaV3.2 (F, HEK-CaV3.2, IC_{50} 8 µM (95% CI: 7.1 to 9.0 µM), Hill coefficient 2.0 (95% CI: 1.5 to 2.6)), CaV3.1 (G, HEK-CaV3.1, IC_{50} 7.5 µM (95% CI: 5.9 to 10.8 µM), Hill coefficient 5.6 (95% CI: lower limit 1.6, upper limit undefined) and CaV3.3 (H, HEK-CaV3.3, IC_{50} 9.9 µM (95% CI: 8.7 to 11.3 µM), Hill coefficient 2.2 (95% CI: 1.6 to 3.0). Current voltage relationships in the absence (ctr, black) and in the presence of 6 µM (blue), 12 µM (orange) and 30 µM (red) englerin A are shown in the lower panels. Data represent means ± SEM (C insert, E-H) and means (C, D, F-H). Numbers of measured cells are indicated in brackets.
Figure 3 – Transcript expression of CaV subunits and TRPCs in HAC15 cells. (A) Representative presentation of transcript expression of voltage-gated calcium (CaV) channel subunits in HAC15 cells. HPRT1 and GAPDH were amplified as controls. Negative and positive controls are shown in Figure S3A. (B) Representative presentation of transcript expression of TRPC channels in HAC15 cells. Positive and negative controls are shown in Figure S3B. (C) Whole cell currents detected from HAC15 cells, extracted at +80 mV and -80 mV during 400 ms voltage ramps spanning from -100 mV to 100 mV, applied every 2 s from a holding potential of 0 mV, plotted versus time. The bar indicates application of 1 µM englerin A (EA). Current-voltage relationship of the net EA-mediated current is shown on the right panel. Data represent means ± SEM (C, left) and means (C, right) of 8 measured cells.

Figure 4 – Pharmacological dissection of CaV currents in HAC15 cells. (A) Representative inward current detected from a HAC15 cell during a 400 ms depolarization step to 0 mV from a holding potential (V_h) of -80 mV. The current-voltage relationship (right) was obtained by voltage steps from -90 mV to +70 mV (10 mV increments, V_h -80 mV). (B-G) Current-voltage relationships of CaV currents in HAC15 cells before (ctr, black) and in the presence (red) of the CaV channel blockers mibefradil (B), Z944 (C), ethosuximide (D), SKF 96365 (E), verapamil (F), verapamil subsequently combined with Z944 (F, blue) and englerin A (G). (H) The dose-response relations revealed an IC_{50} of 2.4 µM EA (95% CI: 1.1 to 5.1 µM) at a Hill coefficient of 2.5 for a maximal inhibition by 54% of the CaV currents in HAC15 cells at 0 mV (red), an IC_{50} of 2.6 µM EA (95% CI: 1.3 to 4.6 µM) at a Hill coefficient of 2.9 for 53% inhibition at 10 mV (grey) and an IC_{50} of 2.3 µM EA (95% CI: 1.2 to 4.5 µM) at a Hill coefficient of 3 for a maximally 60% inhibition at -30 mV (black). Hill coefficient and maximal inhibition had to be pre-estimated and fixed for the sigmoidal fit to obtain a reasonable dose-response relationship curve. (I) Inhibition (in percent) of the currents in B-G at -30 mV (grey bars) and 10 mV (white bars), and the ratio of the inhibition from the
single experiments at -30 mV and 10 mV (red bars). Data represent means ± SEM (A right, B-H), means ± SD (I) and example traces (A, left). Numbers of measured cells are indicated in brackets. Circles in I represent single data points. Inhibition at -30 mV versus 10 mV at each treatment was compared by a paired two-tailed students t-tests with P values indicated above the bars.

**Figure 5 – Effects of englerin A and Z944 on aldosterone release in HAC15 cells.**

Basal (A, C) and 10 nM angiotensin II (Ang II) -induced (B, D) aldosterone release (pg aldosterone / µg protein) from 800,000 initially plated HAC15 cells within 4 hours (A, B) and 24 hours (C, D) incubation with standard medium (control), DMSO (0.1%), englerin A (EA, 6 or 12 µM) and Z944 (1 or 10 µM). Data represent means ± SD. Circles represent single data points. Means were compared by one-way ANOVA tests with post-hoc Dunnett’s multiple comparison test. P values of the statistical comparison between the respective treatment and the control are indicated above the bars.
### Tables

Table 1 - Primers used for PCR

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Table 2 – Primers for cloning the full-length CaV3.2 cDNA from HAC15 cells

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</tr>
<tr>
<td>8</td>
<td>SB_1101 (1101f) ATGACCAGAGGCGCACGAGGCGCGC</td>
<td>1734</td>
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<td></td>
<td>SB_254 (254r) GGCATGGTAGATGCTGTGCA</td>
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</table>
Table 3 - Drugs applied during patch clamp experiments

<table>
<thead>
<tr>
<th>Drug</th>
<th>Final concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>mibefradil</td>
<td>10 µM</td>
<td>Cayman Chemical</td>
</tr>
<tr>
<td>Z944</td>
<td>1, 10 µM</td>
<td>Cayman Chemical</td>
</tr>
<tr>
<td>ethosuximide</td>
<td>10 mM</td>
<td>Cayman Chemical</td>
</tr>
<tr>
<td>SKF 96365</td>
<td>10 µM</td>
<td>Cayman Chemical</td>
</tr>
<tr>
<td>englerin A</td>
<td>0.1, 1, 3, 10, 30, 100 nM and 1, 6, 10, 12, 30 µM</td>
<td>Roth, Merck</td>
</tr>
</tbody>
</table>
Figure 1

A. HEK-TRPC4α + TRPC1

B. HEK-TRPC4α + TRPC1

C. HEK-CaV1.2 + β2 (5)

D. HEK-CaV1.2 + β2 (5)

E. HEK-CaV1.2 + β2

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Figure 2

A. Diagram showing the structure of Flp-CaV3.2 with indications of various experimental conditions.

B. Western blot analysis of hCaV3.2 and Calnexin under different conditions.

C. Graph showing the effects of englerin A on Flp-CaV3.2 activity across different voltage levels.

D. Graph showing the inhibition of Flp-CaV3.2 by englerin A at non-induced levels.

E. Graph showing the inhibition of Flp-CaV3.2 by englerin A at induced levels.

F. Graph showing the IC50 and Hill coefficients for HEK-CaV3.2.

G. Graph showing the IC50 and Hill coefficients for HEK-CaV3.1.

H. Graph showing the IC50 and Hill coefficients for HEK-CaV3.3.
Figure 3

A

HAC15

B

TRPC1 TRPC3 TRPC4 TRPC5 TRPC6 TRPC7

bp

HAC15

C

HAC15 (8)

1 μM EA

current (pA/pF)

-10 0 10 20 30

0 100 200 300 time (s)

1 μM EA

pA/pF

-4 -2 0 2 4

mV

-100 -50 50 100
Figure 4

A  HAC15

5 pA/pF
100 ms

B  10 μM mibefradil (8)

10 μM Z944 (8)

10 mM ethosuximide (7)

10 μM SKF 96365 (9)

F  10 μM verapamil (8)

6 μM englerin A (8)

H  englerin A

I  inhibition (%)

IC_{50} 2.6 μM
Hill 2.9

at 10 mV

IC_{50} 2.4 μM
Hill 2.5

at 0 mV

IC_{50} 2.3 μM
Hill 3.0

at -30 mV

inhibition at -30 mV

ratio at 30 mV

inhibition at 10 mV
Figure 5

A. 4 h basal aldosterone release

B. + 10 nM angiotensin II

C. 24 h basal aldosterone release

D. + 10 nM angiotensin II
Supplemental Materials

Title:
Englerin A inhibits T-type CaV channels at low-micromolar concentrations

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Figure S1 – Original Western Blot of Figure 2B. Western blot of protein lysates using an anti-MYC antibody (left). The membrane was stripped and reprobed with an anti-Calnexin antibody as loading control (right). The boxes indicate the part of the Western Blot shown in Figure 2B.
Figure S2

Figure S2 – Inhibition of T-type CaV channels by 1 µM Z944 and effect of "old" englerin A on CaV3.2. (A) T-type whole-cell currents before (ctr, black) and during application of 1 µM (red) Z944 in HEK cells inducibly expressing CaV3.2 (Flp-CaV3.2, 24 h tetracycline-induced) or stably expressing CaV3.1 (HEK-CaV3.1), CaV3.2 (HEK-CaV3.2) and CaV3.3 (HEK-CaV3.3). (B) Whole cell CaV currents in HAC15 cells before (ctr, black) and during application of 1 µM Z944 (red). (C) CaV3.2 currents before (ctr, black) and during application of 12 µM (orange) "old" englerin A in HEK cells stably expressing CaV3.2 (HEK-CaV3.2). The 12 µM englerin A application solution was prepared in external saline the day before and kept 24 hours at room temperature (RT) before it was used. All currents were recorded during 50 ms voltage ramps spanning from -100 to 100 mV from a holding potential of -80 mV and are means of 5 to 9 cells.
Figure S3

**A** +, NCI-H295R  -, water

RNA from the human adrenal gland carcinoma cell line NCI-H295R (+) and water (–) were used as positive (+) and negative (–) controls for RT-PCR of CaV (A) and TRPC (B) transcripts. Fragments of HPRT1 and GAPDH were amplified as further controls. For TRPC7 (control HPRT1 and GAPDH, B right) mRNA of HEK293 cells (HEK) was used as positive control.

**B** +, NCI-H295R  -, water

+HEK  -, water

**Figure S3** – Positive and negative controls for transcript expression in Figure 3. RNA from the human adrenal gland carcinoma cell line NCI-H295R (+) and water (–) were used as positive (+) and negative (–) controls for RT-PCR of CaV (A) and TRPC (B) transcripts. Fragments of HPRT1 and GAPDH were amplified as further controls. For TRPC7 (control HPRT1 and GAPDH, B right) mRNA of HEK293 cells (HEK) was used as positive control.
**Figure S4**

**Figure S4** – Characterization of CaV currents in the absence and presence of CaV blockers in HAC15 cells. (A-F, H) Inward currents detected from HAC15 cells during 400 ms depolarization steps to -30 mV (left panels) and 10 mV (right panels) from a holding potential...
(V<sub>H</sub>) of -80 mV before (ctr, black) and during (red) incubation with the Ca<sup>2+</sup> channel blockers mibefradil (10 µM; A), Z944 (10 µM; B and F), ethosuximide (10 mM; C), SKF 96365 (10 µM; D), verapamil (10 µM; E), verapamil + Z944 (both 10 µM; E and F, blue) and englerin A (6 µM; H). In E, Z944 was applied on top of verapamil, and in F verapamil on top of Z944. (G) Current-voltage relationships obtained in HAC15 cells by voltage steps from -90 mV to +70 mV (10 mV increments, V<sub>H</sub> -80 mV) before (ctr, black) and in the presence of Z944 (red) with subsequent addition of verapamil (blue). Data represent means ± SEM (G) and means (A-F, H). Numbers of experiments are indicated in brackets.