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Role of voltage-gated calcium channels in potassium-stimulated aldosterone secretion from rat adrenal zona glomerulosa cells

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Abstract

The mineralocorticoid aldosterone plays an important role in the regulation of plasma electrolyte homeostasis. Exposure of acutely isolated rat adrenal zona glomerulosa cells to elevated K^+ activates voltage-gated calcium channels and initiates a calcium-dependent increase in aldosterone synthesis. We developed a novel 96-well format aldosterone secretion assay to rapidly evaluate the effect of known T- and L-type calcium channel antagonists on K^+-stimulated aldosterone secretion and better define the role of voltage-gated calcium channels in this process. Reported T-type antagonists, mibefradil and Ni^{2+}, and selected L-type antagonist dihydropyridines, inhibited K^+-stimulated aldosterone synthesis. Dihydropyridine-mediated inhibition occurred at concentrations which had no effect on rat α1H T-type Ca^{2+} currents. In contrast, below 10 μM, the L-type antagonists verapamil and diltiazem showed only minimal inhibitory effects. To examine the selectivity of the calcium channel antagonist-mediated inhibition, we established an aldosterone secretion assay in which 8Br-cAMP stimulates aldosterone secretion independent of extracellular calcium. Mibefradil remained inhibitory in this assay, while the dihydropyridines had only limited effects. Taken together, these data demonstrate a role for the L-type calcium channel in K^+-stimulated aldosterone secretion. Further, they confirm the need for selective T-type calcium channel antagonists to better address the role of T-type channels in K^+-stimulated aldosterone secretion.

Keywords: T-type; L-type; Voltage-gated; Voltage activated; HVA; LVA; Calcium; Zona glomerulosa; CaV3; CaV1; Alpha1h; Alpha1c; Aldosterone; Amlodipine; Efonidipine; Nifedipine; Diltiazem; Verapamil; Mibefradil; 8Br-cAMP

1. Introduction

By regulating the renal expression of several ion channels and transport proteins, the mineralocorticoid aldosterone plays a critical role in the long-term regulation of plasma electrolyte balance and volume. These processes serve as an important mechanism of blood pressure homeostasis. Recently, mRNA encoding the receptor for aldosterone has also been identified in brain, heart and vascular tissues [1–3]. Clinical and experimental data indicate that aldosterone and its receptor are implicated in CNS, renal and cardiovascular organ damage [4–8].

The principle physiological stimuli of aldosterone secretion are angiotensin II (AII), K^+ and adrenocorticotropic hormone (ACTH). Despite the different second messenger systems used by each of these stimuli, all are dependent upon, or enhanced by, extracellular calcium [9,10]. Exposure of adrenal zona glomerulosa cells to elevated potassium levels depolarizes the membrane potential which activates calcium influx by opening voltage-gated calcium channels (Ca_V) thereby initiating a calcium dependent increase in aldosterone synthesis.

Historically, there has been disagreement about the exact subfamily of Ca_V involved in mediating this K^+-stimulated calcium influx. L-type and T-type calcium channels have been identified in the zona glomerulosa of rat and bovine adrenal glands in both functional and molecular studies, and each has been implicated in regulating K^+-stimulated aldosterone secretion [11–18]. The current consensus in the literature is that T-type calcium channels are the primary regulators of K^+-stimulated aldosterone secretion.

Arguments for the role of T-type channels in regulating K^+-stimulated aldosterone secretion focus on their activation

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at lower membrane potentials, and their ability to sustain calcium influx near these potentials. T-type calcium currents observed in zona glomerulosa cells activate at membrane potentials near −70 mV [15,19–22]. The voltage dependence of T-type current activation and inactivation overlap, indicating that a fraction of these channels will remain conducting when the membrane potential is held between −70 and −40 mV [15,19–22]. Zona glomerulosa cells typically rest at −80 to −95 mV and have been reported to have a membrane potential sensitive to slight alterations in K^+ concentration [15,20,23]. Increases of 1–3 mM K^+ in plasma are sufficient to stimulate aldosterone secretion [24] and likely result in small depolarizations of zona glomerulosa membrane potential. In contrast, L-type channels typically activate near −20 mV [25,26], and are likely to be closed under these conditions. Further support of the T-type channel as mediator of K^+-stimulated aldosterone secretion comes from the sensitivity of this secretion to the relatively T-selective antagonists mibefradil and Ni^{2+} [15,27–30].

Evidence for the role of L-type channels in mediating K^+-stimulated aldosterone secretion comes from several observations. An L-type current has been shown to activate as low as −58 mV in rat zona glomerulosa cells [11], and therapeutic concentrations of the L-selective antagonist nifedipine have been shown to inhibit K^+-stimulated aldosterone secretion [31–34]. However, K^+-stimulated secretion is insensitive to therapeutic concentrations of the L-type specific antagonists diltiazem and verapamil [31,33]. Thus, there are conflicting data regarding which calcium channels are essential for K^+-stimulated aldosterone secretion.

The study of aldosterone secretion in vitro has required acute isolation of zona glomerulosa cells, usually from either bovine or rat adrenal glands. Typically, greater than 50,000 cells are then incubated in 1.5-ml microcentrifuge tubes in the presence or absence of stimulus and test agents. The large number of cells required per data point and the extensive manipulation of tubes necessarily have made studies of large numbers of compounds and/or complete concentration–response curves cumbersome.

In the current studies, we describe a novel aldosterone secretion assay that is run in 96-well format and utilizes a relatively small number of cells per data point. Using this assay, we were able to carry out extensive concentration–response evaluations of both agonists and antagonists of aldosterone secretion. Further, we explored in detail the role of voltage-gated calcium channels in mediating K^+-stimulated aldosterone secretion. These studies establish the importance of L-type calcium channels in this critical pathway of regulating aldosterone secretion.

2. Materials and methods

2.1. Materials

Medium 199 (M0393), 8-bromo-cAMP, $NiCl_2$, verapamil, nifedipine, thapsigargin and fatty acid-free BSA were purchased from Sigma-Aldrich (St. Louis, MO). Aldosterone EIA kits were purchased from Cayman Chemical (Ann Arbor, MI). Aldosterone RIA kits were purchased from Diagnostic Products Corporation (Los Angeles, CA). Collagenase type I and deoxyribonuclease type I were purchased from Worthington Biochemical Corp. (Lakewood, NJ). Diltiazem, mibefradil, amlodipine (racemic mixture and enantiomers) and efonidipine were purified or synthesized at Merck Research Labs. Sprague–Dawley rats were obtained from Taconic (Germantown, NY). All animal procedures were approved by the Institutional Animal Care and Use Committee at Merck Research Labs, West Point, PA.

2.2. Aldosterone secretion assays

Aldosterone secretion assays were performed on freshly isolated cells from male Sprague–Dawley rats weighing 350–500 g using a collagenase dispersion method similar to that previously described [35]. Briefly, rats were anesthetized with 0.7 ml/100 g body weight of a cocktail mix of Ketamine (100 mg/ml), Xylazine (100 mg/ml) and Acepromazine (10 mg/ml), and the adrenal glands removed. Capsules were isolated from the adrenal glands and dissected free of fat and blood vessels. Minced pieces were digested in 2 mg/ml collagenase and 0.3 mg/ml DNase for 1 h at 37°C in a spinner flask with oxygenation. The media used to prepare the cells and for the K^+ stimulation experiments was a modified Medium 199 containing (in mM) 4 K^+, 1.25 Ca^{2+}, 1.2 Mg^{2+}, 4.4 $NaHCO_3$, 10 HEPES and 0.1% fatty acid-free BSA adjusted to pH 7.4 with NaOH. The digested capsules were washed three times and triturated. The cells were then filtered through a 70-μm cell strainer, pelleted and counted. Cell concentration was then adjusted to 2.5×10^5 cells/ml, oxygenated and incubated for at least 2 h at 37°C before experiments. After the incubation, the cells were diluted 1:15 in either modified Medium 199, or 1.2 mM Mg^{2+} in Dulbecco's PBS with or without 1.25 mM Ca^{2+} and centrifuged at $100 \times g$ for 15 min. Cells were resuspended to 40,000–80,000 cells/ml and 125 μl of the cell suspension was added to wells containing 100 μl test compound in appropriate media. The assays were run in a 96-well optical reaction plate designed for thermal cyclers (Applied Biosystems). The plates were oxygenated, capped and placed into a shaking water bath at 37°C. After 10 min, 25 μl of stimulus was added. Each tube was mixed by gentle pipetting, oxygenated, capped and incubated at 37°C for the designated time. After the incubation, the samples were transferred to a Whatman 96-well unifilter microplate and vacuum filtered into a 96-well collection plate to rapidly separate the media from the cells. The media was stored at −20 °C until analyzed in duplicate for aldosterone concentration using either an EIA or RIA.

2.3. Electrophysiology

Whole-cell patch clamp recordings were performed on HEK 293 cells ($V_{holding} = -100$ mV), expressing rat α1H

($Ca_V3.2$) T-type calcium channels [36], with an Axon Instruments (Foster City, CA) model 200B Integrating Patch Clamp amplifier or an Axon Instruments 700A multi-clamp amplifier. Data were digitized (10 kHz) by a Digidata 1320A analog-to-digital converter and analyzed off-line with Axon Instruments software, Clampex 8.2 or later. For recording, patch pipettes (2–4 MΩ series resistance) contained (in mM): 125 $CsMeSO_3$, 10 HEPES, 8 NaCl, 1 $CaCl_2$, 10 EGTA, 2 Mg-ATP, 0.3 GTP, and pH was adjusted to 7.2 with CsOH. Extracellular solution was based on Tyrode solution (in mM): 130 NaCl, 4 KCl, 30 glucose, 25 HEPES, 1 $MgCl_2$, 2 $CaCl_2$, pH adjusted to 7.4 with NaOH. Access resistance was monitored online and was typically <10 MΩ. Baseline T-type calcium currents were elicited by depolarizing from holding to −20 mV for 40 ms, cycling every 10 s. Once stable current amplitudes were observed, an extracellular solution containing a known concentration of compound was perfused in the bath. Percent inhibition was determined from the difference of the peak current prior to compound addition and after steady-state inhibition was reached. Estimated IC_{50} concentrations were calculated by the equation: estimated IC_{50} = ([compound concentration (M) × (100/percent inhibition)] − [compound concentration (M)]) × (1×10^9).

3. Results

3.1. 96-well aldosterone secretion assay development

In order to maximize the data collected from the fewest number of animals, cell density and time course experiments were first performed. Fig. 1 shows the time course of aldosterone secretion by 5000 or 10,000 cells incubated in control (4 mM) or 7 mM K^+. Media were separated from the cells at 20, 40 and 60 min after potassium addition and assayed for aldosterone content. The amount of aldosterone secreted from cells in 4 mM K^+ was consistently below the 40 pg/ml required for detection in the immunoassay standard curves. Stimulation of 5000 cells with 7 mM K^+ for 40 min typically resulted in secreted aldosterone concentrations of 700–2000 pg/ml. Doubling the number of cells approximately doubled the amount of aldosterone produced. Because the signal from 5000 cells is consistently sufficient to reliably detect inhibition after 40 min of stimulation, these conditions were used in subsequent studies.

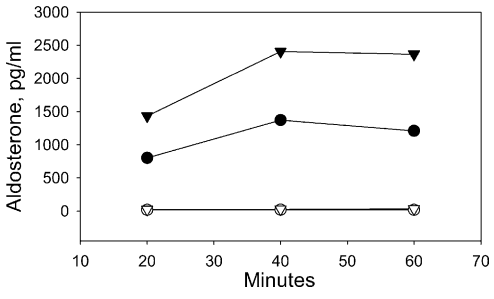

Fig. 1. Time course and cell density effects on K^+-stimulated aldosterone secretion. Either 5000 (circles) or 10,000 (inverted triangles) zona glomerulosa cells were exposed to 4 mM K (open symbols) or 7 mM K (filled symbols) in modified M199 media. Secreted aldosterone was quantitated after 20, 40 and 60 min. Replicate wells are averaged.

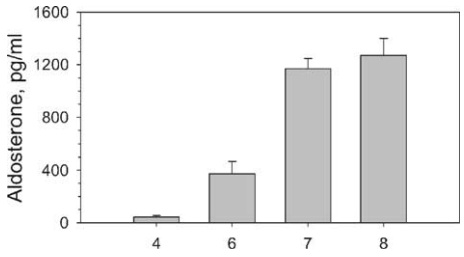

Fig. 2. Optimization of K^+ stimulus for aldosterone secretion. Zona glomerulosa cells were stimulated with different concentrations of K^+ in modified M199 media for 40 min. Secreted aldosterone values are reported as mean ± S.E.M. of replicate wells from a single experiment. Data are representative of two separate experiments.

Previous reports have used depolarizing concentrations of K^+ ranging from 6 to 60 mM to stimulate aldosterone secretion [15,22,30,33,37,38]. One objective of the current studies was to determine the role of T-type Ca^{2+} channels in aldosterone secretion. Since these channels activate at lower membrane potentials than L-type Ca^{2+} channels, we sought to minimize zona glomerulosa depolarization, and therefore, the amount of K^+ used to stimulate secretion. To optimize the final K^+ concentration in the 96-well format assay, we stimulated aldosterone secretion with 4, 6, 7 and 8 mM K^+ (Fig. 2). Stimulating with 7 mM K^+ maximized the secretion response while minimizing the depolarizing signal, and therefore, the potential influence of the high voltage activated L-type channel [15]. In subsequent experiments, K^+ concentrations were 4 and 7 mM, for control and stimulated wells, respectively.

3.2. Non calcium channel antagonist inhibitors of aldosterone secretion

Potassium-stimulated aldosterone secretion is dependent upon the function of several effector proteins following the increase in cytosolic Ca^{2+}. We further validated the miniaturized assay by testing antagonists of K^+-stimulated aldosterone secretion including CaMKII inhibitors and atrial natriuretic peptides. Compounds and peptides showed inhibition with IC_{50}'s similar to those previously reported. (Data not shown.)

3.3. Response to aldosterone secretion stimuli and their dependence on Ca^{2+}

We next confirmed that using only 5000 isolated zona glomerulosa cells would consistently produce measurable secreted aldosterone in response to several stimuli acting through various second messenger pathways in addition to that induced by elevated K^+. These included angiotensin II, thapsigargin (an endoplasmic reticulum calcium ATPase

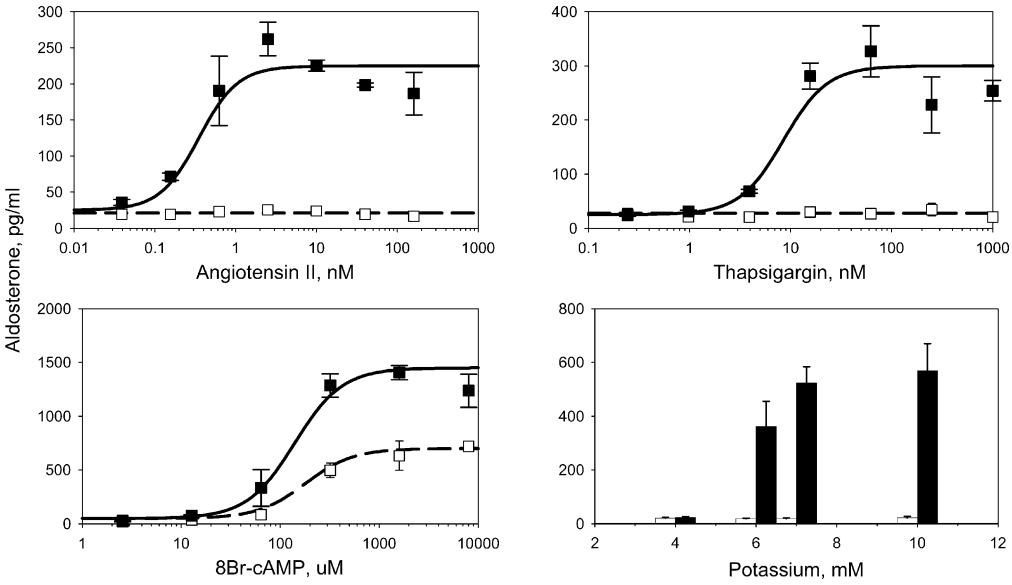

Fig. 3. Aldosterone secretion in response to various stimuli and its dependence on extracellular Ca^{2+}. Zona glomerulosa cells were washed in modified DPBS with (filled symbols) or without (open symbols) 1.25 mM Ca^{2+} and incubated with varying concentrations of the indicated stimulus for 40 min. Secreted aldosterone was quantitated and reported as mean ± S.E.M. from three independent experiments.

inhibitor), 8Br-cAMP (a membrane permeable protein kinase A agonist and mimic of the ACTH stimulus pathway) and K^+. Additionally, the Ca^{2+}-dependence of aldosterone secretion in response to these stimuli was tested by carrying out the assay in the presence or absence of 1.25 mM Ca^{2+}.

The results in Fig. 3 clearly show that all these agonists could promote detectable aldosterone secretion and that all stimuli except 8Br-cAMP are fully dependent on extracellular calcium. Aldosterone secretion stimulated by 8Br-cAMP is significantly enhanced by extracellular calcium but does not require its presence. EC_{50} values obtained in the presence of calcium were 0.31 nM for angiotensin II, 7.0 nM for thapsigargin and 113 μM for 8Br-cAMP. The EC_{50} obtained for 8Br-cAMP in the absence of added calcium was 205 μM.

3.4. Effects of calcium channel antagonists on K^+-stimulated aldosterone secretion

We tested the ability of the relatively T-type calcium channel selective antagonist, mibefradil, to inhibit 7 mM K^+-stimulated aldosterone secretion. As previously reported using other agonists, mibefradil demonstrated concentration-dependent inhibition with IC_{50} of 0.81 μM (Fig. 4). Mibefradil was tested in each experiment as a positive control to determine the precision of the assay and to confirm the quality of cell preparations. The calculated IC_{50} value from the fit of averaged percent of control responses from 29 experiments was similar to that obtained by averaging the IC_{50}'s determined from independent experiments (not shown).

To determine the role of T- and L- type voltage-gated calcium channels in K^+-stimulated aldosterone secretion, complete concentration response curves were obtained for selective L-type antagonists (verapamil, diltiazem, nifedipine and amlodipine), a reportedly mixed T- and L-type antagonist (efonidipine) [39] and another relatively selective T-type antagonist (Ni^{2+}). Results are summarized in Fig. 4. The phenylalkylamine, verapamil, showed no effect below 1 μM, but did inhibit at higher concentrations (IC_{50} = 4.7 μM). The benzothiazepine, diltiazem, showed no inhibition of secretion at concentrations up to 10 μM. The first generation dihydropyridine (DHP), nifedipine, showed potent but incomplete inhibition of aldosterone secretion (IC_{50} = 0.45 μM, maximum inhibition of 67% at 10 μM). In contrast, a third generation DHP, amlodipine, showed potent and complete inhibition of secretion with an IC_{50} of 0.07 μM. The isolated (−) and (+) enantiomers of amlodipine also each showed full inhibition with IC_{50}'s of 0.32 and 6.20 μM, respectively. Efonidipine potently and completely inhibited aldosterone secretion (IC_{50} = 0.04 μM). The relatively selective T-type antagonists Ni^{2+} and the enantiomer of mibefradil were also full inhibitors of secretion with IC_{50}'s of 700 and 0.61 μM, respectively.

This demonstration of potent and complete inhibition of K^+-stimulated aldosterone secretion by an L-type calcium channel selective antagonist suggests the importance of an L-type current for K^+-stimulated aldosterone secretion.

3.5. Selectivity of calcium channel antagonist activity

Both L- and T-type selective compounds fully inhibited K^+-stimulated aldosterone secretion. This leaves the question of whether the inhibition observed is due solely to decreased calcium channel conductance or to inhibition of the aldosterone secretion pathway downstream of Ca^{2+} influx. To determine the selectivity of calcium channel antagonist activity, compounds were tested for their effect on aldos-

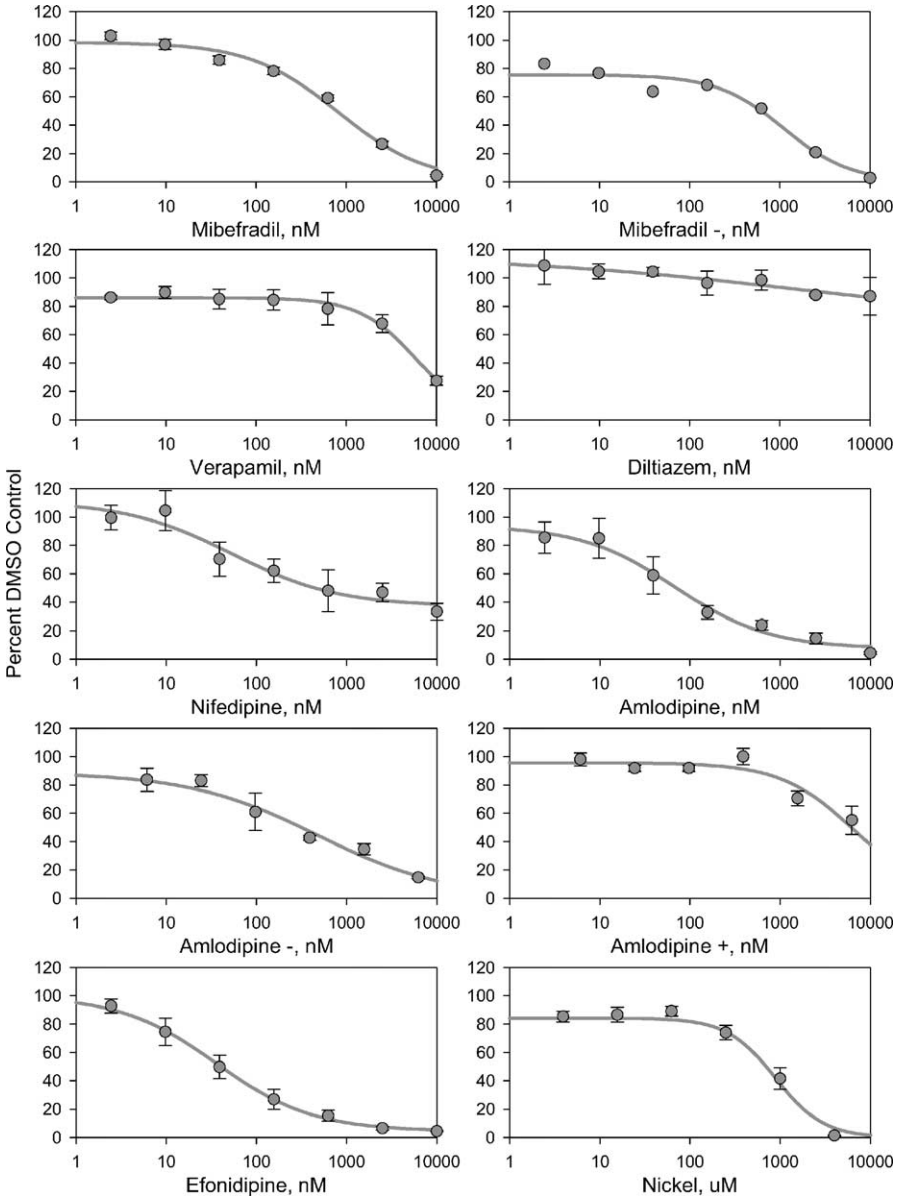

Fig. 4. Effect of calcium channel antagonists on K^+-stimulated aldosterone secretion. The results of three independent experiments (mibefradil $n = 29$) for each compound are averaged and reported as mean ± S.E.M. Compounds and concentration range are indicated for each plot.

terone secretion that occurs independently of calcium influx and thus does not involve a calcium channel. As shown in Fig. 3, 8Br-cAMP stimulates aldosterone secretion in the absence of added extracellular Ca^{2+}. Therefore, any decrease in the secretion of aldosterone observed in the absence of added calcium should be the result of inhibiting the synthetic pathway downstream of calcium influx, not at the calcium channel itself.

Calcium channel antagonists which inhibited K^+-stimulated aldosterone synthesis were thus tested for their ability to prevent 8Br-cAMP-stimulated secretion in the presence and absence of extracellular calcium. Fig. 5 shows these results as well as the effect of the calcium channel antagonists on K^+-stimulated secretion, for comparison. Verapamil shows similar weak inhibition when aldosterone secretion is stimulated with 8Br-cAMP or K^+, suggesting that the inhibition observed with this compound occurs downstream of Ca^{2+} influx. Mibefradil and its enantiomer fully inhibited the 8Br-cAMP-stimulated secretion and were slightly less potent than their inhibition of K^+-stimulated secretion. The dihydropyridine compounds nifedipine, amlodipine and efonidipine showed little or no inhibition of 8Br-cAMP-stimulated secretion in either the absence or presence of added extracellular calcium. The racemic mixture of amlodipine inhibited only at concentrations above 2 μM, greater than 100 times the IC_{50} observed to block K^+-stimulated secretion. Because amlodipine does not inhibit aldosterone synthesis in a nominally calcium-free system, its inhibition of K^+-stimulated synthe-

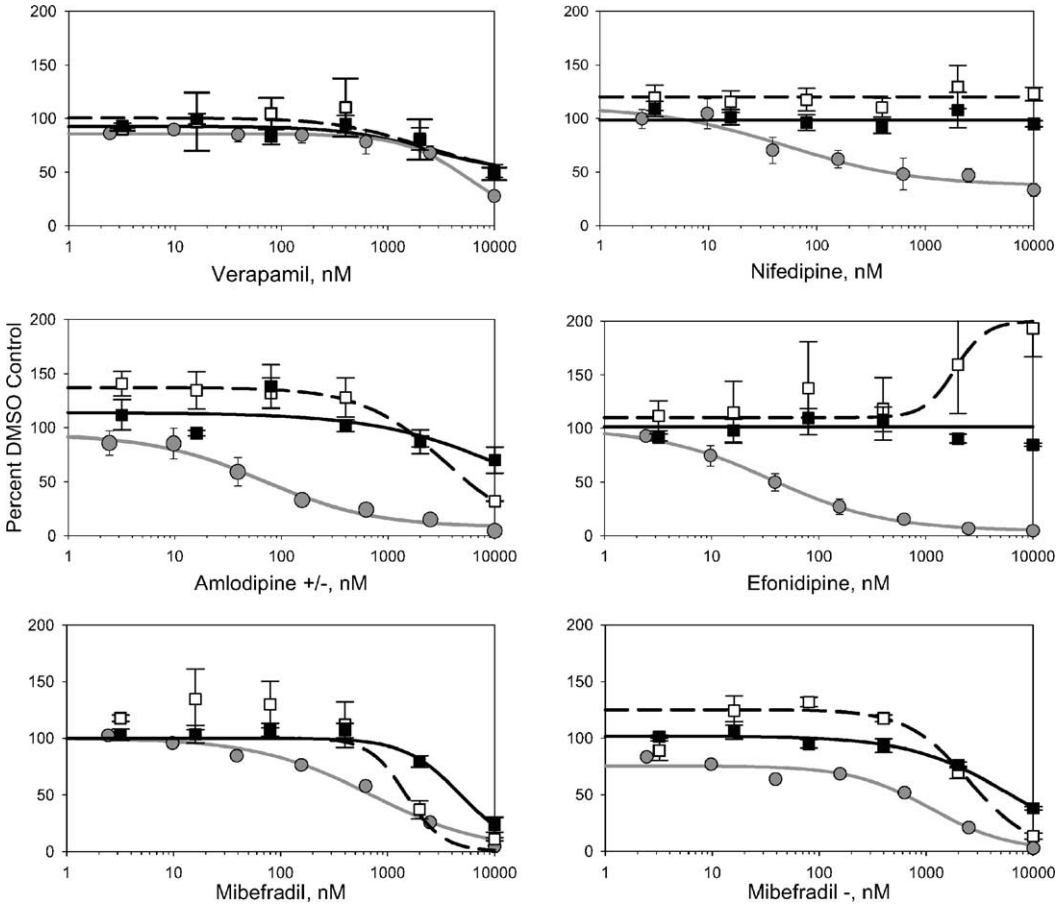

Fig. 5. Effect of calcium channel antagonists on 8Br-cAMP-stimulated aldosterone secretion. Secretion was stimulated in the presence (black squares) or absence (white squares) of 1.25 mM Ca^{2+}. Percent of control data from three independent experiments are plotted as mean ± S.E.M. Data from Figs. 3 and 4 are included (gray circles) for comparison of effects on K^+-stimulated secretion.

sis must be by prevention of calcium influx, and therefore, block of voltage-gated calcium channels.

The aldosterone synthesis stimulated by 8Br-cAMP is enhanced in the presence of extracellular calcium, implying a role for a channel-mediated calcium influx (Fig. 3). The calcium channel antagonists nifedipine, amlodipine and efonidipine do not reduce 8Br-cAMP-stimulated aldosterone secretion in the presence of 1.25 mM Ca^{2+} to the levels seen in the absence of Ca^{2+}. This suggests that the calcium influx required for the maximal 8Br-cAMP-stimulated aldosterone secretion observed in Fig. 3 is not mediated by dihydropyridine-sensitive L-type channels under these conditions.

3.6. Inhibition of T-type currents

While the 8Br-cAMP experiments show that amlodipine and efonidipine do not inhibit aldosterone secretion downstream of calcium in flux, they do not confirm the specificity of calcium channel antagonist activity on L-type versus T-type currents. In fact, efonidipine has been reported to show T-type antagonist activity [39]. Therefore, we determined the ability of selected compounds to inhibit heterologously expressed T-type calcium channel currents. Rat α1H ($Ca_V3.2$) was expressed in HEK 293 cells and membrane current inhibition was assessed by repetitive depolarizations to −20 mV from a holding potential of −100 mV. Representative current traces are depicted in Fig. 6 . The average percent inhibition of peak current for 1 μM mibefradil was 66 ± 1% ($n = 4$); for 25 μM amlodipine 63 ± 3% ($n = 3$) and for 20 μM efonidipine 25 ± 2% ($n = 3$). The estimated IC_{50} values are 0.52 μM for mibefradil, 15 μM for amlodipine and 60 μM for efonidipine. These data reveal that amlodipine and efonidipine are poor inhibitors of T-type calcium currents compared to their ability to inhibit K^+-stimulated aldosterone secretion. In contrast, amlodipine and efonidipine inhibit L-type currents with IC_{50}'s of 0.001 [40] and 0.10 μM [39], respectively. Taken together, these data suggest that these dihydropyridines inhibit aldosterone secretion by blocking L-type calcium currents.

4. Discussion

4.1. Assay development

Prior to this report, most studies of aldosterone secretion have used a large number of primary cells per data point. We

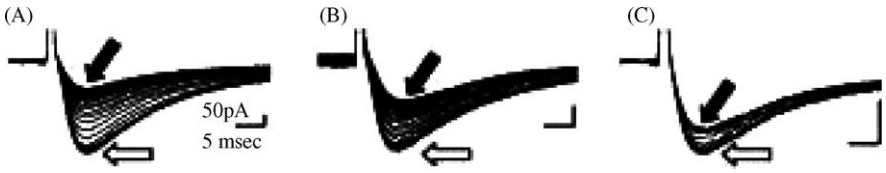

Fig. 6. Inhibition of rat α1h currents by aldosterone secretion antagonists. Example recordings showing the extent of inhibition by (A) mibefradil (1 μM), (B) amlodipine (25 μM) and (C) efonidipine (20 μM). Open arrows represent stable baseline amplitude of peak currents. Filled arrows represent steady-state peak amplitudes following inhibition by application of compound. Capacitance transients were blanked out.

have developed a versatile 96-well format aldosterone secretion assay using only 5000 acutely dissociated rat adrenal zona glomerulosa cells per data point. This format allows much higher throughput than previously published methods and is adaptable to studying both stimulation and inhibition pathways. EC_{50} values obtained for Ang II and 8Br-cAMP were similar to those previously reported [38,41], while thapsigargin was more potent [42]. Our results show that angiotensin II-stimulated aldosterone secretion is dependent on the presence of extracellular Ca^{2+}, in contrast with a previous report [41]. A possible reason for this difference is that they used 20–40-fold more cells per data point and had detectable aldosterone synthesis in the absence of stimulus. The lower number of cells used here may still be stimulated in the absence of calcium, but the amount of aldosterone produced remains below detection. This miniaturized assay was used in the current studies to examine the role of voltage-gated calcium channels in regulating the aldosterone secretion response to elevated K^+ concentrations.

4.2. Detecting inhibition of aldosterone secretion downstream of calcium influx

Removal of Ca^{2+} from the media minimizes the influence of any plasma membrane associated calcium channel. Therefore, comparing aldosterone secretion stimulated by K^+ in the presence of Ca^{2+} to that stimulated by 8Br-cAMP in the absence of Ca^{2+} distinguishes compounds which selectively inhibit K^+-stimulated Ca^{2+} influx from compounds that inhibit the downstream signaling and/or synthetic pathways. Comparison of effects under these two conditions is important since inhibition at any of the several steps required for aldosterone synthesis would be detected. Additionally, comparing stimulated aldosterone secretion avoids reliance on background levels of synthesis and enables fewer cells to be used per data point.

Verapamil and mibefradil have both been shown to inhibit aldosterone secretion stimulated by cAMP analogs or ACTH [15,34,43–45]. We show that verapamil inhibits aldosterone secretion stimulated by K^+ in the presence of Ca^{2+} and 8Br-cAMP in the absence of calcium with similar potencies. Thus, verapamil may inhibit calcium channels, but inhibition of aldosterone secretion at 10 μM likely occurs downstream of the calcium influx. Consistent with our observed inhibition of aldosterone synthesis downstream of calcium influx, Schiebinger et al. [34] detected elevated levels of corticosterone but not aldosterone in stimulated zona glomerulosa cells exposed to verapamil. This indicates that verapamil did not prevent the initial stimulus for aldosterone synthesis, but inhibited the downstream conversion of corticosterone to aldosterone.

Additional information regarding the role of calcium channels in stimulated aldosterone secretion can be obtained by comparing 8Br-cAMP-stimulated aldosterone secretion in the presence and absence of added Ca^{2+}. Compounds, which inhibit stimulated secretion in the presence of Ca^{2+} to the levels observed in the absence of Ca^{2+}, can be interpreted as selectively inhibiting the calcium channel(s) required for maximal aldosterone secretion stimulated by 8Br-cAMP. None of the compounds tested in the current studies showed this pattern, indicating that DHP-sensitive calcium channels are not involved in the Ca^{2+}-dependent 8Br-cAMP aldosterone secretion pathway.

4.3. Role for L-type channels in K^+-stimulated aldosterone secretion

There is disagreement in the literature with respect to which family of voltage-gated calcium channels is responsible for conducting the K^+-mediated aldosterone secretion stimulus signal. However, the current consensus is that T-type calcium channels are critical. The failure of diltiazem at 10 μM and the high concentrations of verapamil required to inhibit aldosterone secretion are consistent with a role for T-type, not L-type calcium channels, and agree with previous reports [31]. As discussed above, higher concentrations of verapamil likely inhibit K^+-stimulated aldosterone secretion downstream of calcium influx, not via blockade of L-type calcium channels, further supporting this hypothesis. In contrast, the inhibition of K^+-stimulated aldosterone secretion by amlodipine with an IC_{50} of 0.07 μM, a compound with an IC_{50} for T-type currents of 15 μM, clearly suggests a role for L-type calcium channels. Additionally, we show Efonidipne is also a very weak blocker of T-type current (IC_{50} of 60 μM) versus its inhibition of K^+-stimulated aldosterone secretion (IC_{50} of 0.03 μM). Further, efonidipine does not inhibit 8Br-cAMP-stimulated secretion in the absence of added calcium, indicating its effects are specific to calcium channels. The results with amlodipine and efonidipine are in stark contrast with the prevailing view that T-type calcium channels mediate K^+-stimulated aldosterone secretion.

A possible explanation for these apparently conflicting conclusions regarding the role of L-type calcium channels could be that the different structural classes of L-type antagonists clearly have different mechanisms of inhibition (rate-dependence or state-dependence) as well as binding sites [46,47]. Even within the dihydropyridine class, compounds can have widely variable physiological effects [48]. Therefore, it is possible that the compounds previously used to conclude there is no role for L-type channels do not have the appropriate characteristics to observe potent inhibition of K^+-stimulated aldosterone secretion.

The lack of truly selective T-type calcium channel antagonists also prevents an unambiguous conclusion regarding the role of T-type channels. Both nickel [49] and mibefradil [50] are capable of inhibiting L-type currents within several-fold of the concentrations which inhibit T-type currents. Comparison of the observed 700 μM IC_{50} for Nickel in our aldosterone secretion assay with the published 13 μM IC_{50} for inhibition of the α1H current [51] suggests that the observed inhibition by Ni^{2+} is not mediated by blockade of T-type calcium currents. Previous reports have used 200–1000 μM concentrations of Nickel in similar assays and observed only partial inhibition [28–30], consistent with our results. Likewise, the observed 0.81 μM IC_{50} for mibefradil is above the reported 0.14 μM for a T-type mediated event [52]. The lack of inhibition observed with these compounds at concentrations which selectively inhibit T-type calcium currents prevents decisive identification of the relevant calcium channel.

Our results cannot directly address one piece of data that does not support a role for L-type calcium channels in K^+-stimulated aldosterone secretion by Barrett et al. [30]. They showed that the toxin, ω-Aga-IIIA, reduced L-type current amplitude but did not prevent aldosterone secretion. However, the relevant L-type current may not have been identified electrophysiologically. Subsequent studies showed that the predominant L-type mRNA in rat adrenal zona glomerulosa cells is α1D or $Ca_V1.3$ [14]. This channel has been reported to activate at a threshold of −45 mV [53,54], and as low as −65 mV in other tissues [55]. Assuming the presence of these low threshold activating α1D channels, the ramp protocol used by Barrett et al. to isolate the L-type current would not allow clear separation of the T-type current from α1D currents. In fact, Varnai et al. have used a modified ramp protocol to demonstrate an L-type current in rat zona glomerulosa cells, which activates at −58 mV [11]. Additionally, the α1D current recorded in hair cells was only partially inhibited by nifedipine [55], which is consistent with our and other observations of incomplete inhibition of K^+-stimulated aldosterone secretion by nifedipine [31,33,34]. Future experiments will determine the ability of ω-Aga-IIIA to antagonize α1D.

It is difficult to reach conclusions about the role of various calcium channels when comparing electrophysiology results to secretion experiments. There are significant differences in the cell preparations used to determine the electrophysiological properties of adrenal zona glomerulosa cells versus detecting stimulated aldosterone secretion. It is possible that, during the 24–72 h incubation times required to achieve high-quality electrophysiology results [21], the expression profile of calcium channel subunits or regulatory proteins may have changed from that in the acutely isolated cells which are used to detect stimulated aldosterone secretion. This could affect both the kinetics and pharmacology of the relevant currents.

While the results described here clearly identify a role for L-type calcium channels in K^+-stimulated aldosterone secretion, they do not exclude a role for T-type channels. Further experiments with a truly T-selective compound, knock-out animals or siRNA will be required in order to define the role of T-type calcium channels in K^+-stimulated aldosterone secretion.

In summary, we have developed a high throughput aldosterone secretion assay using acutely isolated rat adrenal zona glomerulosa cells. Using this assay, we identified both agonists and antagonists of aldosterone secretion at multiple points of distinct second messenger pathways. We have also developed an aldosterone secretion assay that functions in nominally calcium-free media that serves as a functional counter-screen for inhibitors of calcium influx. Using these assays, we have demonstrated that the dihydropyridine amlodipine potently inhibits K^+-stimulated secretion and that similar concentrations have no effect on 8Br-cAMP-stimulated secretion in the presence or absence of extracellular calcium. We have also confirmed that amlodipine, at the concentration which inhibit aldosterone secretion, does not inhibit the predominantly expressed T-type calcium channel in zona glomerulosa cells. These data demonstrate a role for dihydropyridine-sensitive calcium channels in K^+-stimulated aldosterone secretion from acutely dissociated rat adrenal zona glomerulosa cells. Further, the inconclusive data with Ni^{2+} and mibefradil show a need for truly selective T-type calcium channel antagonists to better address the role of T-type channels in mediating K^+-stimulated aldosterone secretion.

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