Table 2 The distribution of the swollen lymph nodes detected by thoracic CT in patients with cardiac sarcoidosis

Case (Age/Sex)	1 (60/M)	2 (63/M)	3 (51/F)	4 (49/F)	5 (51/F)	6(55/M)	7(44/M)	8(59/F)
#1 Highest Mediastinal	−	−	−	−	−	−	−	−
#2 Upper Paratracheal	−	+	−	−	+	−	−	−
#3 Pre-vascular and Retrotracheal	+	+	−	+	−	−	−	−
#4 Lower Paratracheal	+	+	+	+	+	+	+	−
#5 Subaortic	−	+	+	−	+	−	+	−
#6 Paraaortic	+	+	+	+	+	−	+	−
#7 Subcarinal	−	−	+	−	−	−	+	−
#8 Paraesophageal	+	−	+	−	−	−	+	−
#9 Pulmonary ligament	−	−	−	−	−	−	−	−
#10 Hilar	+	−	+	−	−	−	−	−
#11 Interlobar	−	−	−	−	−	−	+	−
#12 Lobar	−	−	−	−	−	−	−	−
#13 Segmental	−	−	−	−	−	−	−	−
#14 Subsegmental	−	−	−	−	−	−	−	−

Inhibitory Effect of Efonidipine on Aldosterone Synthesis and Secretion in Human Adrenocarcinoma (H295R) Cells

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Abstract: Targeting aldosterone synthesis and/or release represents a potentially useful approach to the prevention of cardiovascular disease. Aldosterone production is stimulated by angiotensin II (Ang II) or extracellular K^+ and is mediated mainly by Ca^{2+} influx into adrenal glomerulosa cells through T-type calcium channels. We therefore examined the effects of efonidipine, a dual T-type/L-type Ca^{2+} channel blocker, on aldosterone secretion in the H295R human adrenocarcinoma cell line; 100 nmol/L Ang II and 10 mmol/L K^+ respectively increased aldosterone secretion from H295R cells 12-fold and 9-fold over baseline. Efonidipine dose-dependently inhibited both Ang II- and K^+-induced aldosterone secretion, and nifedipine, an L-type Ca^{2+} channel blocker, and mibefradil, a relatively selective T-type channel blocker, similarly inhibited Ang II- and K^+-induced aldosterone secretion, but were much less potent than efonidipine. Efonidipine also lowered cortisol secretion most potently among these drugs. Notably, efonidipine and mibefradil also significantly suppressed Ang II- and K^+-induced mRNA expression of 11-β-hydroxylase and aldosterone synthase, which catalyze the final two steps in the aldosterone synthesis, whereas nifedipine reduced only K^+-induced enzyme expression. These findings suggest that efonidipine acts via T-type Ca^{2+} channel blockade to significantly reduce aldosterone secretion, and that this effect is mediated, at least in part, by suppression of 11-β-hydroxylase and aldosterone synthase expression.

Key Words: adrenal gland, aldosterone, Angiotensin II, efonidipine, potassium, T-type calcium channels

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Aldosterone plays an important role in the pathophysiology of hypertension, cardiac hypertrophy, and heart failure. Recent clinical trials have shown that inhibition of the biologic actions of aldosterone using a mineralocorticoid receptor antagonist alone or in conjunction with an angiotensin converting enzyme inhibitor or an angiotensin II (Ang II) receptor antagonist is reported to be useful add-on therapy in hypertensive patients and reduces the incidence of cardiovascular events among patients with heart failure.[1–3]

Aldosterone is synthesized in the zona glomerulosa of the adrenal gland from which it is secreted in a Ca^{2+}-dependent manner in response to stimuli such as Ang II, adrenocorticotropic hormone (ACTH), and increased extracellular K^+.[4] Bovine and human glomerulosa cells express L-type and T-type voltage-dependent Ca^{2+} channels,[5] though recent studies suggest that T-type channel activity is more closely related to aldosterone production than L-type channel activity.[6,7] Ang II activates both T-type and L-type Ca^{2+} channels indirectly by inhibiting K^+ channel activity, which leads to plasma membrane depolarization.[8] Likewise, membrane depolarization leading to T-type channel activity are also caused by small elevations in extracellular K^+.[9] Because of their biophysical properties (ie, a low activation threshold and slow inactivation kinetics) T-type Ca^{2+} channels are able to mediate sustained Ca^{2+} influx and thus sustained aldosterone production.[10]

Efonidipine is a dihydropyridine Ca^{2+} channel blocker that voltage clamp experiments have shown to exert an inhibitory effect on both L-type and T-type Ca^{2+} currents in guinea-pig heart.[11] The inhibitory effect of efonidipine on T-type channel activity has also been observed in rat cardiomyocytes[12] and in exogenous channels expressed in Xenopus oocytes, where efonidipine was found to act on the α1H and α1G subtypes of T-type Ca^{2+} channels.[13]

Selective T-type Ca^{2+} channel blockers currently are not widely used. The clinical use of mibefradil, a relatively selective T-type Ca^{2+} channel blocker, was considered about 10 years ago, but its clinical use was limited because of its severe interaction with the enzyme P4503A4.[14] On the other hand, previous studies of metabolic pathway and pharmacokinetics of efonidipine did not show either significant interaction with other drugs or severe adverse effects, so efonidipine has been accepted as a safe and promising drug in clinical use.[15,16] Our aim in the present study was to compare the effects of efonidipine, a dual L-type/T-type Ca^{2+} antagonist, with those of mibefradil and nifedipine, an L-type channel antagonist, on the synthesis and secretion of aldosterone.

MATERIALS AND METHODS

Reagents

Efonidipine was kindly provided by Nissan Chemical Industries, Ltd (Tokyo, Japan). A 1:1 mixture of DMEM/Ham's F-12 medium (DMEM/F12), penicillin-streptomycin solution,

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mibefradil, and nifedipine were purchased from Sigma-Aldrich Co (St. Louis, MO). UltroserSF was from Ciphergen (Fremont, CA). Insulin-transferrin-sodium selenite Plus (ITS) was from BD Bioscience (Bedford, MA). Ang II was from Peptide institute, Inc. (Minoh, Japan).

Cell Culture

The NCI-H295R human adrenocortical cell line (H295) was obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM/F12 medium containing 2% UltroserSF, 1% ITS, and antibiotics (penicillin and streptomycin). The cells were maintained at 37°C under a humid atmosphere of 95% air/5% CO_2 as previously described.[17,18]

Assay of Steroid Secretion

Steroid secretion was assayed essentially as previously described except with a minor modification to take gene expression into account.[19] Briefly, 0.2×10^6 cells were plated in 6-well plates and incubated for 48 hours, after which the medium was replaced with fresh medium containing 0.2% UltroserSF, and the cells were incubated for an additional 24 hours. This medium was then replaced with medium containing the Ang II (100 nmol/L) or KCl (10 mmol/L) with or without the indicated concentration of a Ca^{2+} channel blocker and incubated for 48 hours. During that period, the medium was refreshed once after 24 hours. Levels of aldosterone in the conditioned media were assayed using a SPAC-S aldosterone RIA kit (TFB Co., Tokyo, Japan). Cortisol levels were also measured using a commercially available EIA kit (Oxford Biomedical Research, Oxford, MI).

RT-PCR Analysis

H295R cells were plated to a density of 0.2×10^6 in 6-well plates and incubated for 48 hours, after which the medium was replaced with low-serum medium containing 0.2% UltroserSF, and the cells were then treated with Ang II (100 nmol/L) or KCl (10 mmol/L) with or without the indicated concentration of Ca^{2+} channel blocker for 24 hours. The cells were then harvested, and the total cellular RNA was extracted using the isothiocyanate-acid phenol chloroform method (TRIzol; Invitrogen, Carlsbad, CA). After quantifying the extracted RNA based on absorbance at 260 nm, 500-ng aliquots were reverse transcribed using a SuperScript Choice System (Invitrogen). The expression of aldosterone synthetic enzymes and Ca^{2+} channel proteins was evaluated by RT-PCR analysis using the primer sets shown in Table 1. All primers were synthesized by Sigma Genosys (Ishikari, Japan). To discriminate PCR products that might arise from possible chromosomal DNA contaminants, PCR primer pairs were designed so that one half of the primer hybridized to the 3′ end of one exon and the other half to the 5′ end of the adjacent exon. Real-time PCR was carried out using a QantiTect SYBR Green PCR system (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, after which the identities of the amplified products were verified by partial sequencing.

Statistical Analyses

Differences between groups were analyzed using ANOVA followed by post hoc Dunnett analysis (StatView; SAS Institute Inc., Cary, NC). Values of $P < 0.05$ were considered significant.

RESULTS

Inhibitory Effect of Efonidipine on Aldosterone Secretion

The effect of efonidipine on aldosterone secretion was compared with those of two other Ca^{2+} channel blockers: nifedipine, a dihydropyridine that efficiently blocks L-type channels,[20] and mibefradil, a benzimidazolyl-substituted tetraline derivative that reportedly inhibits T channels at concentrations 10-fold lower than is necessary for L-type channel inhibition.[21] Under control conditions, 100 nmol/L Ang II and 10 mmol/L K^+ respectively increased aldosterone secretion from H295R cells 12-fold (0.88 ± 0.09 pmol/mg protein/h (mean $\pm$ SD)) and 9-fold (0.62 ± 0.08 pmol/mg protein/h) over baseline (0.07 ± 0.01 pmol/mg protein/h). As shown in Figure 1A, efonidipine inhibited Ang II-induced aldosterone secretion in a dose-dependent manner, completely abolishing secretion at a concentration of 3 μmol/L. Aldosterone secretion was also inhibited by micromolar concentrations of nifedipine or mibefradil, but efonidipine was significantly ($P < 0.01$) more potent than either nifedipine or mibefradil (Fig. 1A, inserted table).

Efonidipine inhibited K^+-induced aldosterone secretion even more potently than it did Ang II-induced secretion, completely blocking secretion at a concentration of 0.1 μmol/L (Fig. 1B). K^+-induced aldosterone secretion was also inhibited at lower concentrations by nifedipine and mibefradil, but again efonidipine was significantly more potent (Fig. 1B, inserted table).

In similar fashion, efonidipine inhibited both Ang II- and K^+-induced secretion of cortisol, as did nifedipine and mibefradil (Fig. 2). Again efonidipine was significantly more potent than either of the other two antagonists (Fig. 2, inserted tables).

TABLE 1. Sequence of Selected Oligonucleotide Used as RT-PCR Primers

Target	Forward	Reverse
CYP11B1	5′-GCTGCACCATGTGCTGAAA	5′-TGGGGACAAGGTCAGCAAG
CYP11B2	5′-CCAGCTGGGACATTGGTAC	5′-CTGGCCTTGCTATTGACAAG
CACNA1C	5′-GGGCTAAAGCATGTGGTTC	5′-TGTAGTTGCCCTTGCATTCC
CACNA1H	5′-CAACCCAAGTCGCTGGAC	5′-CTGGTTCCACCTGTCCTTG

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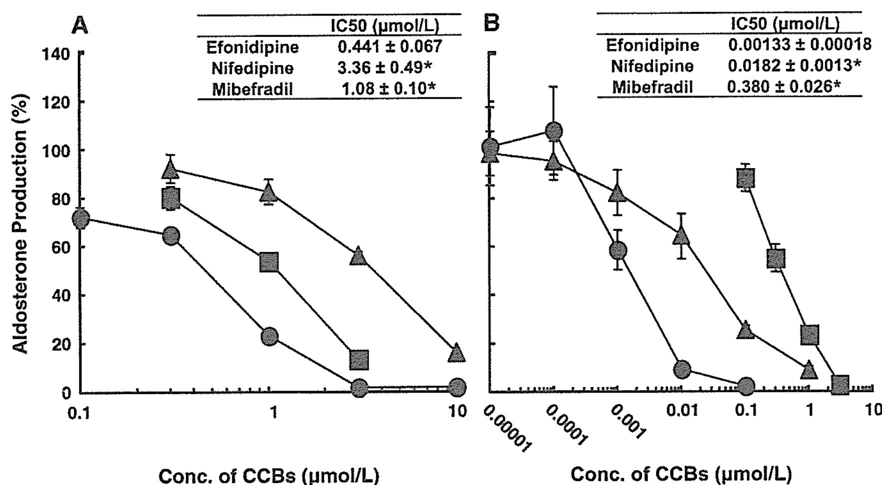

FIGURE 1. Inhibitory effect of efonidipine (circles), nifedipine (triangles), and mibefradil (squares) on aldosterone secretion from H295R cells stimulated with 100 nmol/L Ang II (A) or 10 mmol/L KCl (B). x-axis represents concentrations of calcium channel blockers (CBBs). Symbols depict mean percentages ± SD of the values obtained without inhibitors in six independent experiments. Statistical analysis of IC50 values (inserted table) entailed analysis of variance followed by Dunnett's post hoc analysis; *$P < 0.01$ versus efonidipine. The baseline, Ang II-evoked, and K^+-evoked aldosterone levels were 0.07 ± 0.01 (mean ± SD), 0.88 ± 0.09, and 0.62 ± 0.08 pmol/mg protein/h, respectively.

Down-regulation of mRNAs Encoding Steroidogenic Enzymes by Efonidipine

To better understand the mechanism of the inhibitory effect of efonidipine on aldosterone and cortisol secretion, we next investigated its effects on the mRNA expression of enzymes involved in aldosterone and cortisol synthesis. The final two steps in the synthesis of aldosterone are catalyzed by 11-β-hydroxylase (P450c11), which is encoded by *CYP11B1* and converts 11-deoxycorticosterone to corticosterone, and aldosterone synthase (P450c18), which is encoded by *CYP11B2* and converts corticosterone to aldosterone. To measure aldosterone synthase mRNA correctly, we took special care to construct PCR primer sets that selectively detected *CYP11B2* or *CYP11B1* (Table 1), as the two genes are highly homologous. As shown in Figure 3A, Ang II and 10 mmol/L K^+ respectively induced 26-fold and 20-fold increases in the levels of aldosterone synthase mRNA. Efonidipine suppressed Ang II-induced increases in the level of the transcript to 85% ($P < 0.05$) and 54% ($P < 0.01$) of control at concentrations of 0.3 μmol/L and 3 μmol/L, respectively, and completely blocked K^+ (10 mmol/L)-induced expression at a concentration of 0.1 μmol/L (Fig. 3B). Mibefradil (3 μmol/L) also significantly inhibited Ang II- and K^+-induced expression of aldosterone synthase mRNA, but as with secretion it was less potent than efonidipine. Nifedipine, by contrast, did not inhibit Ang II-induced expression of aldosterone synthase mRNA, though it did inhibit K^+-induced expression.

Efonidipine also suppressed Ang II- and K^+-induced expression of 11-β-hydroxylase mRNA (Figs. 3C and 3D), as did mibefradil, though with less potency than efonidipine. As with aldosterone synthase, nifedipine had no effect on Ang II-induced expression of 11-β-hydroxylase mRNA, but it did significantly inhibit K^+-induced expression (Figs. 3C and 3D).

Effect of Efonidipine on Expression of Ca^{2+} Channel Proteins

Finally, we assessed the expression of T-type and L-type voltage-dependent Ca^{2+} channels, which are the target molecules of the drugs used in this study. Previous studies have shown that, like adrenal glomerulosa cells, H295R cells

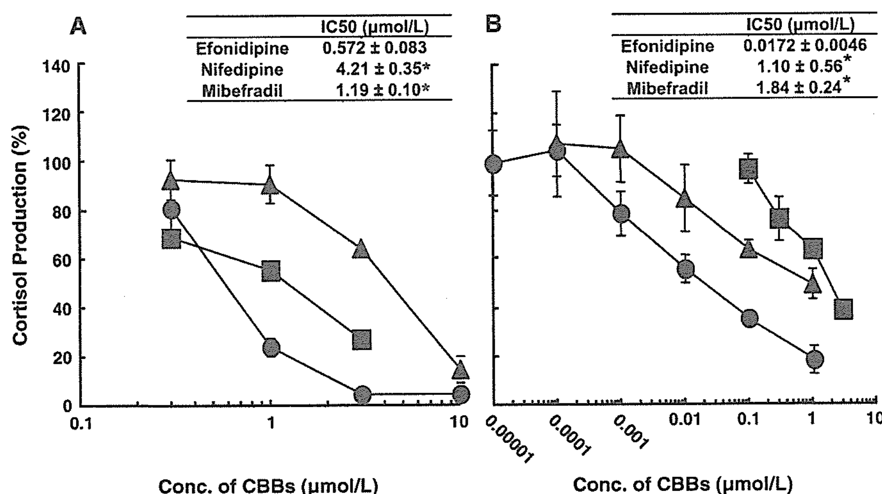

FIGURE 2. Inhibitory effect of efonidipine (circles), nifedipine (triangles), and mibefradil (squares) on cortisol secretion from H295R cells stimulated with 100 nmol/L Ang II (A) or 10 mmol/L KCl (B). x-axis represents concentrations of calcium channel blockers (CBBs). Symbols depict mean percentages ± SD of the values obtained without inhibitors in six independent experiments. Statistical analysis of IC50 values (inserted table) entailed analysis of variance followed by Dunnett's post hoc analysis; *$P < 0.01$ versus efonidipine. The baseline, Ang II-evoked, and K^+-evoked cortisol levels were 12.1 ± 0.5 (mean ± SD), 19.5 ± 1.9, and 27.9 ± 0.5 pmol/mg protein/h, respectively.

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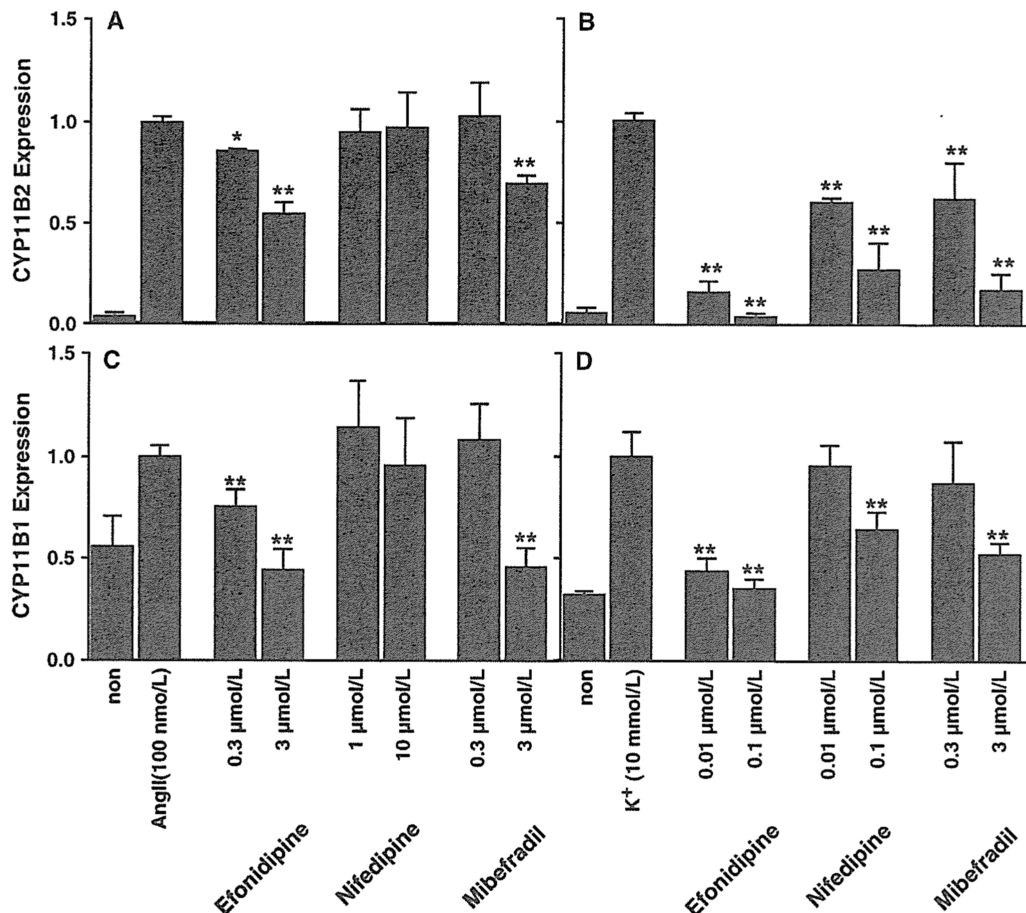

FIGURE 3. Effect of Ca^{2+} channel inhibitors on Ang II-induced (A, C) and K^+-induced (B, D) expression of steroidogenic enzymes. Expression of aldosterone synthase (A, B) and 11-β-hydroxylase (C, D) mRNAs were normalized to that of GAPDH mRNA and expressed relative to values obtained without inhibitors. *Columns* and *bars* represent the means ± SD (n = 6 in for each test group). Statistical analysis entailed analysis of variance followed by Dunnet's post hoc analysis; *$P < 0.05$, **$P < 0.01$ versus a group without inhibitors.

express T-type and L-type channels.[6,22] We found that transcription of *CACNA1H*, which encodes the α1H subunit (a T-type α subunit), was augmented by both Ang II and K^+, but that expression was unaffected by any of the three Ca^{2+} channel blockers tested (Figs. 4A and 4B). Likewise, Ca^{2+} channel blockade had no effect on Ang II-induced transcription of *CACNA1C*, which encodes the α1C subunit (an L-type α subunit) (Fig. 4C). By contrast, K^+-induced expression α1C mRNA was significantly reduced by 55% in the presence of 0.1 μmol/L efonidipine (Fig. 4D). Mibefradil had a similar effect but was less potent, attenuating expression of α1C mRNA by 38% at a concentration of 3 μmol/L. Nifedipine had no significant effect on the expression α1C mRNA.

DISCUSSION

We have shown that the dihydropyridine Ca^{2+} channel blocker efonidipine exerts an inhibitory effect on aldosterone synthesis and secretion in a human adrenocortical cell line (H295R). Given that efonidipine has a higher affinity for T-type than L-type Ca^{2+} channels,[13] this effect is consistent with earlier studies showing that two other T-type channel blockers, mibefradil and tetrandrine,[10,23] inhibit aldosterone secretion from bovine adrenal glomerulosa cells. As a dihydropyridine, however, the chemical structure of efonidipine is quite different from that of mibefradil, a benzimidazolyl-substituted tetraline derivative, or tetrandrine, an abis-bezylistoqu alkaloid, which likely accounts for its greater potency. over, H295R cells harbor both *CACNA1H* and *CACN* which encode α subunits of the T-type and L-type Ca^{2+} cha respectively (Fig. 4). In these cells, efonidipine more stro inhibited aldosterone secretion than did nifedipine, again s gesting that T-type Ca^{2+} channel activity is more closely relat to aldosterone secretion than L-type channel activity.

We found that efonidipine suppresses both Ang II- an K^+-induced aldosterone secretion, but that it blocked the latter at much lower concentrations than the former. This difference almost certainly reflects differences in their respective mechanisms

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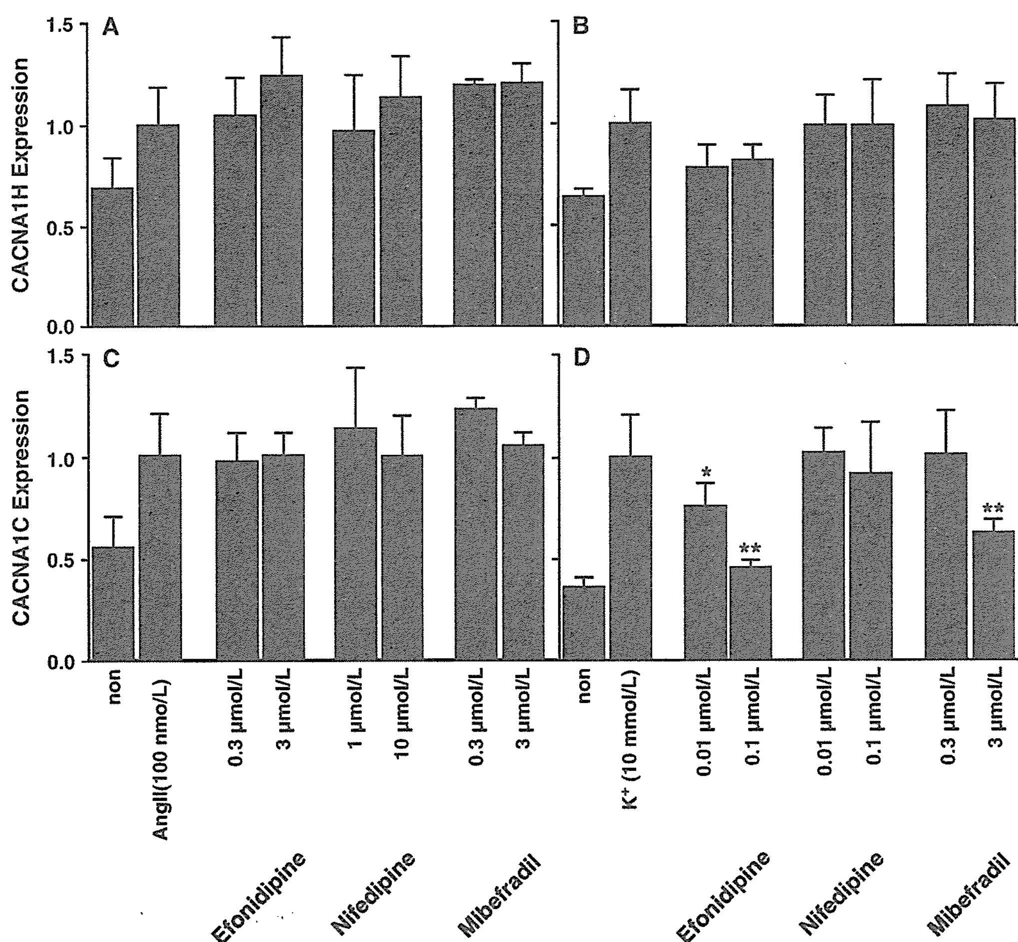

FIGURE 4. Effect of chemicals Ca^{2+} channel inhibitors on Ang II-induced (A, C) and K^+-induced (B, D) expression of Ca^{2+} channel proteins. Expression of α1H (A, B) and α1C (C, D) subunit mRNA was normalized to that of GAPDH mRNA and expressed relative values obtained without inhibitors. *Columns* and *bars* represent the means ± SD (n = 6 in for each test group). Statistical analysis entailed analysis of variance followed by Dunnet's post hoc analysis; $*P < 0.05$, $**P < 0.01$ versus a group without inhibitors.

of action. An extracellular K^+ concentration of 10 mmol/L would be expected to cause a small membrane depolarization that would be sufficient to induce T-type Ca^+ channel opening, but would have little or no effect on intracellular Ca^{2+} stores.[9] By contrast, acting via its type 1 receptor, Ang II elicits not only membrane depolarization but also activates the protein kinase C pathway, which can mediate release of Ca^{2+} from intracellular stores.[24–26] Both of these pathways could potentially lead to increased aldosterone synthesis, as aldosterone synthase is transcriptionally up-regulated via the Ca^{2+}-calmodulin kinase pathway.[27,28]

Earlier reports have looked at the effect of blocking T-type Ca^{2+} channels on aldosterone secretion, but the effect on expression of steroidogenic enzymes has not been investigated. Our present findings indicate that at a concentration that completely inhibits evoked aldosterone secretion, efonidipine also almost completely suppressed Ang II- and K^+-evoked transcription of *CYP11B1 and CYP11B2*, which respectively encode 11-β-hydroxylase and aldosterone synthase, the enzymes that catalyze the final two steps in the synthesis of aldosterone. Further efonidipine did not affect the *CACNA1H* expression level as shown in Figure 4. It thus appears that the inhibitory effect of efonidipine on aldosterone secretion results, at least in part, from transcriptional inhibition of 11-β-hydroxylase and aldosterone synthase expression. Further supporting this idea is our finding that efonidipine inhibited secretion of both aldosterone and cortisol from H295R cells with about the same potency (Figs. 1 and 2). This likely reflects that fact that 11-β-hydroxylase catalyzes not only synthesis of corticosterone from 11-deoxycorticosterone but also the synthesis of cortisol from 11-deoxycortisol.

To date, there have been no published reports on the effect of efonidipine on plasma aldosterone levels in vivo. Upon oral administration of 40 mg of efonidipine to humans, the plasma efonidipine level reaches about 0.025 µmol/L,[15] which our present findings indicate is sufficient to suppress aldosterone synthesis and secretion induced by high K^+. In that regard, we observed that 40 mg of efonidipine significantly

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reduced plasma aldosterone concentration in humans, whereas nilvadipine, L-type calcium channel blocker increased it. However both agents increased plasma renin activity (unpublished observation).

We found that nifedipine, an L-type Ca^{2+} channel blocker, also reduces aldosterone secretion and potassium-induced CYP enzyme expression, which is in contrast to the classic view of no inhibitory effect of L-type channel blockers on aldosterone production.[10,23] Although the precise mechanism for the inhibitory effect of nifedipine on aldosterone production is not clear at present, possible explanations are that influx of Ca^{2+} through L-type channels is involved in aldosterone synthesis or that nifedipine partially blocks T-type channels. The reason for the discrepancy may be due to the difference in the experimental design and cells used between the present and previous experiments. In earlier works, primary cultured bovine adrenal cells were treated with nifedipine for 4 to 5 hours, but in this study we used human adrenocortical tumor cells, and the gene expression levels were evaluated after 24-hour treatment with the calcium channel blockers and the aldosterone production was measured after 48-hour treatment. Further studies are needed to confirm the inhibitory effects of L-type calcium channel blocker on aldosterone and cortisol production.

It is also noteworthy that although K^+-induced aldosterone secretion was blocked by a much lower concentration of efonidipine than was needed to block Ang II-induced secretion, mibefradil inhibited the two responses with equal potency. This might reflect mibefradil's ability to block multiple K^+ channels in addition to the T-type Ca^{2+} channel, which could attenuate the membrane depolarization caused by an elevation in extracellular K^+.[29,30]

In summary, we found that efonidipine, a dihydropyridine Ca^{2+} channel antagonist that blocks both L-type and T-type channels, significantly reduces aldosterone secretion from adrenocortical H295R cells, an effect that was mediated, at least in part, by suppression of 11-β-hydroxylase and aldosterone synthase expression. Its potent effect on steroidogenesis offers a possibility of distinct therapeutic benefit of L-type/T-type dual blocker on the aldosterone control in comparison with the L-type calcium channel blockers in clinical use.

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