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European Journal of Pharmacology 584 (2008) 424–434
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Blockade of T-type voltage-dependent Ca^{2+} channels by benidipine, a dihydropyridine calcium channel blocker, inhibits aldosterone production in human adrenocortical cell line NCI-H295R

Osamu Akizuki [1], Atsushi Inayoshi [1], Tetsuya Kitayama, Kozo Yao, Shiro Shirakura, Katsutoshi Sasaki, Hideaki Kusaka, Masahiro Matsubara *

Pharmaceutical Research Center, Kyowa Hakko Kogyo Co., Ltd., Shizuoka 411-8731, Japan

Received 10 September 2007; received in revised form 25 January 2008; accepted 6 February 2008
Available online 12 February 2008

Abstract

Benidipine, a long-lasting dihydropyridine calcium channel blocker, is used for treatment of hypertension and angina. Benidipine exerts pleiotropic pharmacological features, such as renoprotective and cardioprotective effects. In pathophysiological conditions, the antidiuretic hormone aldosterone causes development of renal and cardiovascular diseases. In adrenal glomerulosa cells, aldosterone is produced in response to extracellular potassium, which is mainly mediated by T-type voltage-dependent Ca^{2+} channels. More recently, it has been demonstrated that benidipine inhibits T-type Ca^{2+} channels in addition to L-type Ca^{2+} channels. Therefore, effect of calcium channel blockers, including benidipine, on aldosterone production and T-type Ca^{2+} channels using human adrenocortical cell line NCI-H295R was investigated. Benidipine efficiently inhibited KCl-induced aldosterone production at low concentration (3 and 10 nM), with inhibitory activity more potent than other calcium channel blockers. Patch clamp analysis indicated that benidipine concentration-dependently inhibited T-type Ca^{2+} currents at 10, 100 and 1000 nM. As for examined calcium channel blockers, inhibitory activity for T-type Ca^{2+} currents was well correlated with aldosterone production. L-type specific calcium channel blockers calciseptine and nifedipine showed no effect in both assays. These results indicate that inhibition of T-type Ca^{2+} channels is responsible for inhibition of aldosterone production in NCI-H295R cells. Benidipine efficiently inhibited KCl-induced upregulation of 11-β-hydroxylase mRNA and aldosterone synthase mRNA as well as KCl-induced Ca^{2+} influx, indicating it as the most likely inhibition mechanism. Benidipine partially inhibited angiotensin II-induced aldosterone production, plus showed additive effects when used in combination with the angiotensin II type I receptor blocker valsartan. Benidipine also partially inhibited angiotensin II-induced upregulation of the above mRNAs and Ca^{2+} influx inhibitory activities of benidipine for aldosterone production. T-type Ca^{2+} channels may contribute to additional benefits of this drug for treating renal and cardiovascular diseases, beyond its primary anti-hypertensive effects from blocking L-type Ca^{2+} channels.

Keywords: Benidipine; Aldosterone; T-type Ca^{2+} channel

1. Introduction

Benidipine, a long-lasting dihydropyridine calcium channel blocker, is used for the treatment of hypertension and angina (Kitakaze et al., 1999; Yao et al., 2006). Benidipine predominantly elicits vasodilatation of coronary and peripheral arteries by blocking Ca^{2+} influx via L-type (high threshold) voltage-dependent calcium channels. Benidipine exerts pleiotropic pharmacological features, such as renoprotective and cardioprotective effects (Hayashi et al., 2003; Kawata et al., 1997; Kitakaze et al., 1999; Yao et al., 2006; Yue et al., 2001). However, the mechanism by which benidipine has additional beneficial effects on end-organ damage is poorly defined. Recently, electrophysiological analysis of *Xenopus* oocytes expressing the exogenously

* Corresponding author. Pharmaceutical Research Center, Kyowa Hakko Kogyo Co., Ltd., 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731, Japan. Tel.: +81 55 989 2039; fax: +81 559 86 7430.
E-mail address: masahiro.matsubara@kyowa.co.jp (M. Matsubara).
[1] Osamu Akizuki and Atsushi Inayoshi contributed equally to this work.

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doi:10.1016/j.ejphar.2008.02.001

introduced Cav3.1 subtype of T-type (low threshold) voltage-dependent Ca^{2+} channel demonstrated that benidipine also inhibits T-type Ca^{2+} channels (Furukawa et al., 2005). This has raised the possibility that the additional benefits of benidipine might be due to inhibition of T-type Ca^{2+} channels.

Aldosterone, an antidiuretic hormone, is produced by the zona glomerulosa of the adrenal cortex in response to increased potassium or angiotensin II (Struthers and MacDonald, 2004). Although aldosterone plays an important role in regulating electrolyte composition by promoting sodium retention and potassium excretion, recent basic and clinical studies have indicated pathophysiological roles of aldosterone (Pitt et al., 1999, 2003; Struthers and MacDonald, 2004). Excess aldosterone causes endothelial, myocardial and renal dysfunction, leading to cardiovascular diseases, such as heart failure (Pitt et al., 1999, 2003; Struthers and MacDonald, 2004).

In adrenal glomerulosa cells, aldosterone synthesis is dependent on the increase in intracellular Ca^{2+} (Spat and Hunyady, 2004). Increased Ca^{2+} induces mRNA expression of 11-β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2), which convert 11-deoxycorticosterone to corticosterone, and corticosterone to aldosterone, respectively (Bird et al., 1995; Clyne et al., 1996; Yagci and Müller, 1996). Adrenal glomerulosa cells express both L-type and T-type Ca^{2+} channels, however T-type Ca^{2+} channels, rather than L-type Ca^{2+} channels, play an essential role in aldosterone production (Barrett et al., 1995; Rossier et al., 1996). Calcium channel blockers that block T-type Ca^{2+} channels, such as mibefradil and tetrandrine, inhibit aldosterone production (Rossier et al., 1993, 1998), while the L-type specific calcium channel blocker nifedipine only slightly affects it, even at high concentration (Rossier et al., 1996). However, it has not been determined whether or not benidipine inhibits aldosterone production in adrenal glomerulosa cells.

In this study, effect of benidipine on aldosterone production and T-type Ca^{2+} channels using a human adrenocortical cell line NCI-H295R was investigated. Also, effects of other dihydropyridine calcium channel blockers, such as efonidipine (T-type/L-type calcium channel blocker), cilnidipine (N-type/L-type calcium channel blocker) and nifedipine (L-type calcium channel blocker) were examined (Furukawa et al., 1999, 2005). Previous studies showed that NCI-H295R cells abundantly expressed the Cav3.2 (α1H) subtype of T-type Ca^{2+} channel and produced aldosterone in response to KCl or angiotensin II (Bird et al., 1993; Lesouhaitier et al., 2001). Activity of T-type Ca^{2+} channels in NCI-H295R cells was confirmed by patch clamp techniques (Lesouhaitier et al., 2001), indicating that these cells would be suitable for this study.

2. Materials and methods

2.1. Materials

Benidipine and valsartan were synthesized in our laboratories. Cilnidipine (Fujirebio, Tokyo, Japan) and efonidipine (Zeria, Tokyo, Japan) were extracted from tablets. The following materials were purchased from the company indicated: nifedipine (Sigma-Aldrich, Saint Louis, MO, USA); mibefradil (Sigma-Aldrich); angiotensin II (Sigma-Aldrich); calciseptine (Peptide Institute, Osaka, Japan). Dihydropyridine calcium channel blockers, mibefradil and valsartan, were dissolved in dimethyl sulfoxide at a concentration of 0.1 M and stored at −20 °C before use. Final concentration of dimethyl sulfoxide in the assay system described below was 0.1% v/v. Calciseptine was dissolved in water at a concentration of 100 μM and stored at −20 °C until use.

2.2. Cell culture

NCI-H295R cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 2.5% v/v heat-inactivated fetal bovine serum (Invitrogen), 10 mg/ml insulin, 5.5 mg/ml transferrin, 0.0067 mg/ml selenite (INSULIN-TRANS-SEL-G, Invitrogen), 1 mg/ml linoleic acid (Sigma-Aldrich), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cultures were maintained in 5% CO_2 at 37 °C in culture flasks. Cells were detached with trypsin–EDTA solution (Invtirogen). After the cells were washed, they were seeded in assay plates or dishes, then used for subsequent experiments. To examine mRNA expression or aldosterone production, growth medium was replaced with the assay medium (DMEM/F12 containing 0.094 mg/ml linoleic acid, 100 units/ml penicillin and 100 μg/ml streptomycin).

2.3. Measurement of aldosterone production

Confluent cells (3×10^5 cells/well) in collagen I-coated 24-well plates were treated with the indicated concentrations of calcium channel blockers for 10 min, then stimulated with 10 mM KCl or 100 nM angiotensin II for 24 h. Amount of aldosterone in supernatant was determined using a commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.4. Measurement of mRNA expression level using quantitative real-time polymerase chain reaction (PCR)

Confluent cells (3×10^5 cells/well) in collagen I-coated 24-well plates were treated with the indicated concentrations of calcium channel blockers for 10 min, then stimulated with 10 mM KCl for 24 h or 100 nM angiotensin II for 8 h. Total RNA was extracted from cells with RNeasy (Qiagen, Hilden, Germany) and QIA shredder (Qiagen). Any remaining DNA was digested using a RNase-free DNase Set (Qiagen). Superscript First-strand III Synthesis System (Invitrogen, NY, USA) was used for first-strand cDNA synthesis in a total volume of 20 μl from 0.3 μg of total RNA with oligo (dT) priming. All primers and probes were synthesized at Invitrogen and Operon Biotechnologies (Tokyo, Japan). Primers used were: CYP11B1, forward primer 5′-GGA GAC ACT AAC CCA AGA GGA CAT-3′ and reverse primer 5′-ACG TGA TTA GTT GAT GGC TCT GAA-3′; CYP11B2, forward primer 5′-GAC ACT AAC TCA AGA GGA CAT AAA G-3′ and reverse primer 5′-CTG

GCC TTG CTA TTT GAC AAG-3′; β-actin, forward primer 5′-TGA GCG CGG CTA CAG CTT-3′ and reverse primer 5′-TCC TTA ATG TCA CGC ACG-3′. Probes used were: CYP11B1, 5′-AAG ATG GTC TAC AGC TTC ATA TTG AGG CC-3′; CYP11B2, 5′-CTC CTC ACT TTC AGA GCG ATT AAC TAG TC-3′; β-actin, 5′-ACC ACC ACG GCC GAG CGG-3′. Probes were labeled with a reporter fluorescent dye FAM (6-carboxy-fluorescein) at the 5′-end and a fluorescent dye quencher Tamura (6-carboxy-tetramethyl-rhodamine) at the 3′-end. Quantitative PCR was performed in a reaction mixture (30 μl) consisting of the above first-strand cDNA as template, TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), forward and reverse primers (final 1 μM each), probe (final 0.2 μM) and distilled water, monitored with an ABI PRISM 7700 detection system (Applied Biosystems). PCR conditions were 2 min at 50 °C, 10 min at 90 °C, with 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Expression levels of CYP11B1 and CYP11B2 mRNA were normalized with β-actin mRNA.

2.5. Measurement of intracellular Ca^{2+}

Ca^{2+} response was assessed using fluo-3-AM in conjunction with a fluorometric imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale, CA, USA). Loading of Ca^{2+} indicator was achieved by exposing the cells to FLIPR calcium 3 assay kit dye solution (Molecular Devices) in Hanks's balanced salt solution (Sigma-Aldrich) for 60 min. Confluent cells (3×10^4 cells/well) in collagen I-coated 384-well plates were pretreated with calcium channel blockers for 10 min. Cells were immediately placed on the FLIPR, then stimulated with 10 mM KCl or 100 nM angiotensin II. Fluorescence change was monitored at an excitation wavelength of 488 nm and emission wavelength of 540 nm.

2.6. Electrophysiological recording

NCI-H295R cells were dissociated by digestion with 0.25% trypsin plus 1 mM EDTA for 2 min, then diluted 7-fold with

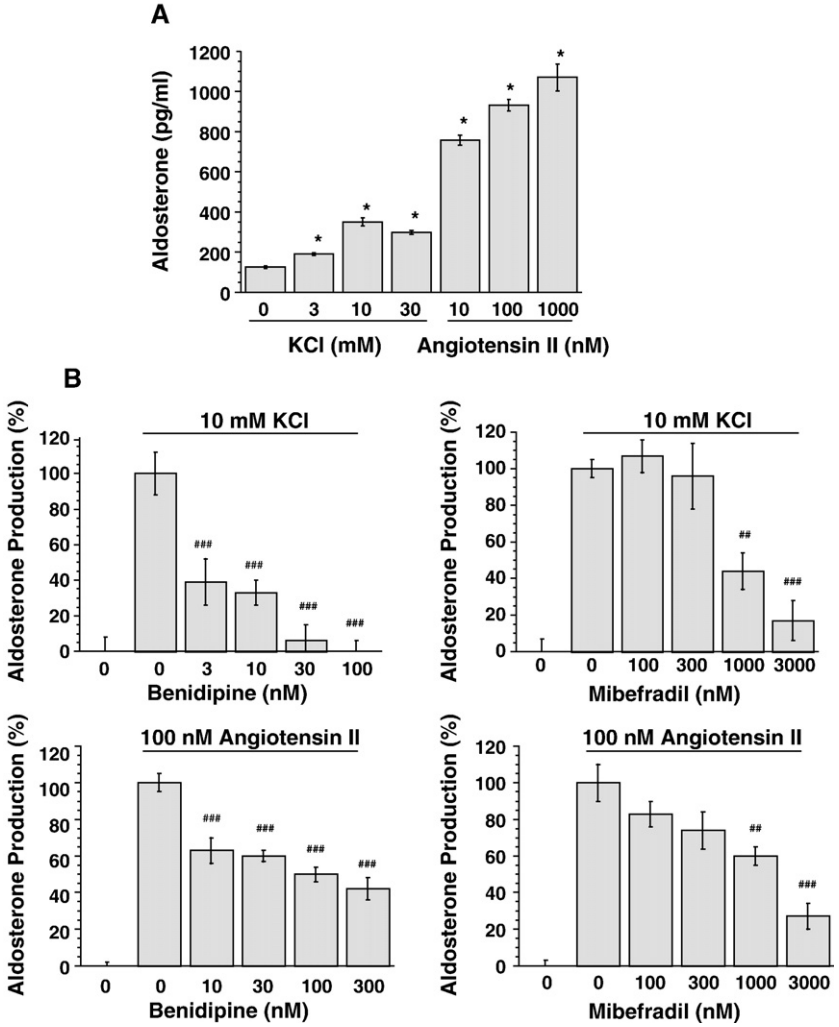

Fig. 1. Effect of benidipine and mibefradil on KCl- or angiotensin II-induced aldosterone production in NCI-H295R cells. (A), NCI-H295R cells were stimulated with the indicated concentrations of KCl or angiotensin II for 24 h. Amount of aldosterone produced in the medium was determined by ELISA. $^{*}P<0.05$ compared to non-stimulated group (Steel test). (B), NCI-H295R cells were pretreated with the indicated concentrations of benidipine or mibefradil for 10 min prior to stimulation with 10 mM KCl or 100 nM angiotensin II for 24 h. Production of aldosterone is shown as % of control. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared to the control group (Dunnett test). Data represent mean±S.E.M. of six determinations in two separate experiments.

growth medium. After transferring 1×10^4 cells/ml to 35-mm culture dishes containing 12-mm diameter glass coverslips, cells were cultured for 1 to 4 days in growth medium before electrophysiological recordings. Recordings were made with standard whole cell voltage-clamp techniques (Hamill et al., 1981). Tetraethylammonium (TEA)-based bath solution for recording of Ca^{2+} current consisted of 160 mM TEA-Cl, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose, adjusted to pH 7.4 with TEA-OH. Cells were generally maintained in the standard bath solution (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES, adjusted to pH 7.4 with NaOH) until seal formation, at which time the standard bath solution was switched to the TEA-based bath solution. The pipette solution consisted of 110 mM CsCl, 3 mM $MgCl_2$, 3 mM Mg-ATP, 10 mM EGTA and 10 mM HEPES, adjusted to pH 7.4 with CsOH. Patch pipettes were fabricated from glass capillary tubes (GDC1.5, Narishige) by a vertical two-stage electrode puller (PP-83, Narishige). Pipette resistance was 3–5 MΩ when filled with the pipette solution. Membrane currents were recorded using an Axopatch 1D (Axon Instruments, Inc., Foster City, CA, USA) and pClamp6 data acquisition software (Axon Instruments, Inc.). Data were digitized at 5 kHz, filtered at 1 kHz and analyzed using Origin 6.0 software. All experiments were performed at room temperature (20–25 °C). Cells were relatively homogenous in size with cell membrane capacitance of 13.2 ± 0.7 pF (n = 55). Cell membrane

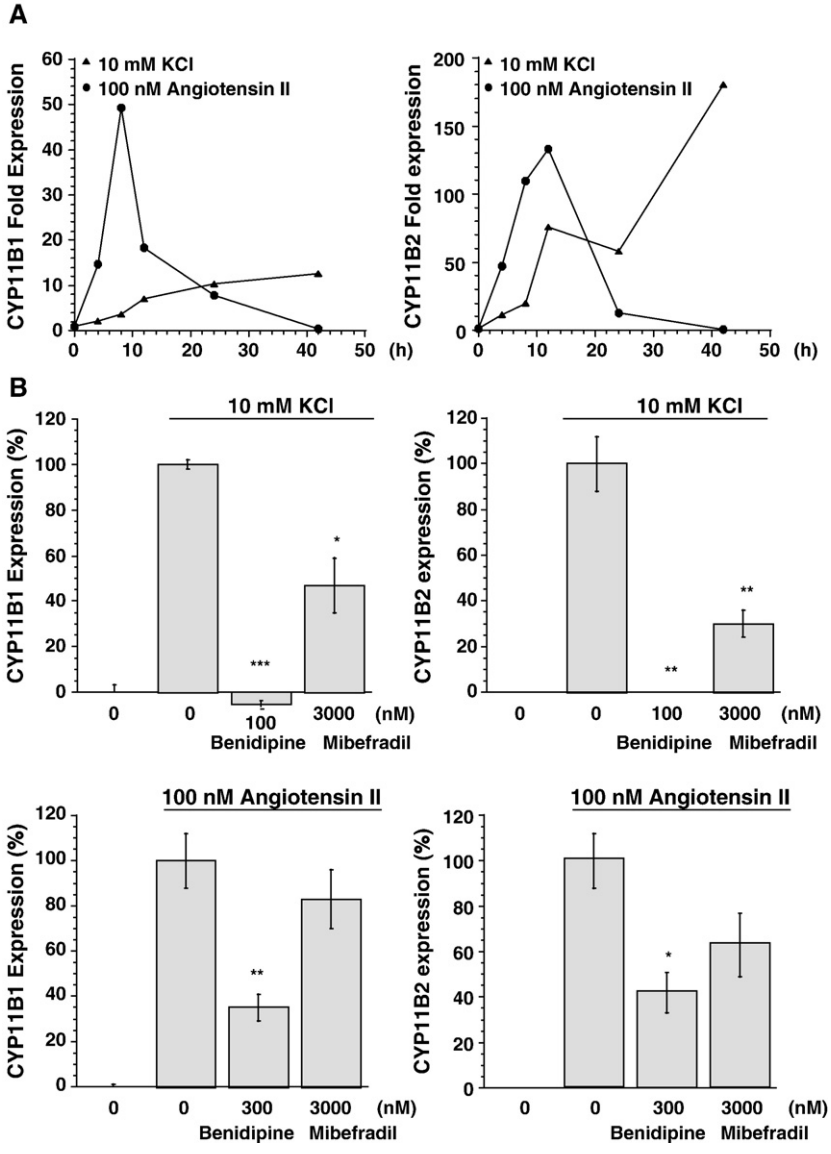

Fig. 2. Effect of benidipine and mibefradil on KCl- or angiotensin II-induced CYP11B1 and CYP11B2 mRNA expression in NCI-H295R cells. (A), NCI-H295R cells were stimulated with 10 mM KCl or 100 nM angiotensin II for the indicated times. mRNA levels of indicated genes were analyzed using real-time RT-PCR. Expression levels of indicated mRNAs were normalized to β-actin. Time course data is shown as one representative result of two independent experiments. Data represent mean of two determinations. (B), NCI-H295R cells were pretreated with benidipine (100 or 300 nM) or mibefradil (3000 nM) for 10 min prior to stimulation with 10 mM KCl for 24 h or 100 nM angiotensin II for 8 h. Expression levels are shown as % of control. *P<0.05, **P<0.01, ***P<0.001 compared to the control group (Student's t-test). Data represent means ± S.E.M. of three determinations.

capacitance was compensated and series resistance was compensated without oscillations. The cell was voltage clamped at a holding potential of −60 mV and depolarized or hyperpolarized as indicated. When holding at a more negative potential than −60 mV, it was difficult to maintain a tight seal for a long period. Therefore, a prepulse prior to a test pulse was applied for hyperpolarization to a more negative potential in order to recover from inactivation. Current amplitude was measured by the peak of inward current to the current remaining at the end of a 200 ms test step.

2.7. Statistical analysis

Statistical analysis was performed using statistical analysis software (SAS, version 9.1.3, SAS Institute, Cary, NC, USA). The Aspin–Welch test or Student's *t*-test following the *F*-test was used for analysis of differences between two groups. Multiple comparisons among treatment groups were assessed by one-way analysis of variance, followed by the Dunnett's test or Steel test. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Benidipine inhibits aldosterone production, mRNA expression of CYP11B1 and CYP11B2, plus calcium response in KCl- or angiotensin II-stimulated NCI-H295R cells

Stimulation with KCl (3–30 mM) or angiotensin II (10–1000 nM) concentration-dependently produced aldosterone in

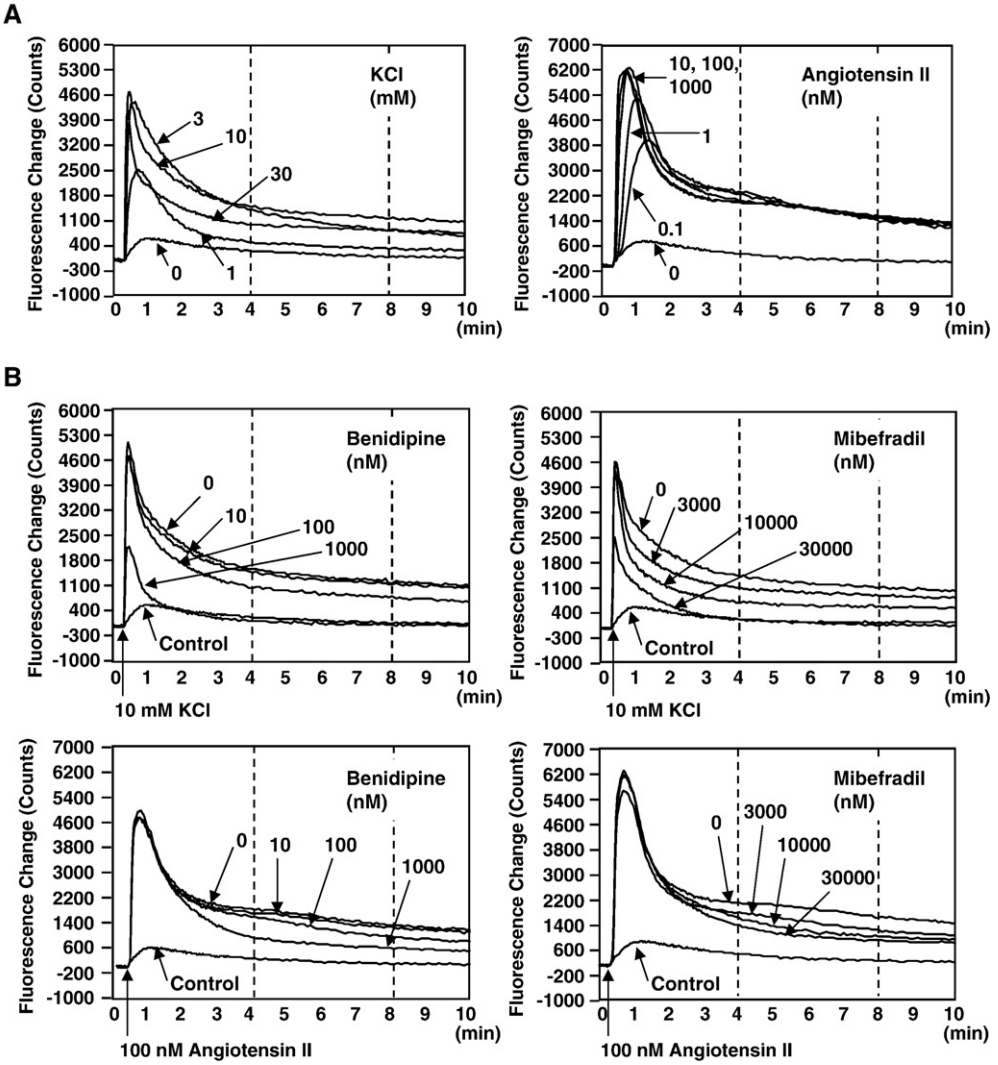

Fig. 3. Effect of benidipine and mibefradil on KCl- or angiotensin II-induced Ca^{2+} response in NCI-H295R cells. (A), KCl- or angiotensin II-induced Ca^{2+} response in NCI-H295R cells was assessed using fluo-3-AM in conjunction with a fluorometric imaging plate reader. Fluo-3-loaded cells were stimulated with KCl (1, 3, 10 and 30 mM) or angiotensin II (0.1, 1, 10, 100 and 1000 nM). Fluorescence change of fluo-3 was monitored. Data represent the average value of fluorescence measured in four determinations. (B), Effect of benidipine and mibefradil on evoked Ca^{2+} responses. Fluo-3-loaded cells were treated with benidipine (10, 100 and 1000 nM) or mibefradil (10,000 and 30,000 nM), then stimulated with 10 mM KCl or 100 nM angiotensin II. Data represent mean value of fluorescence measured in four determinations.

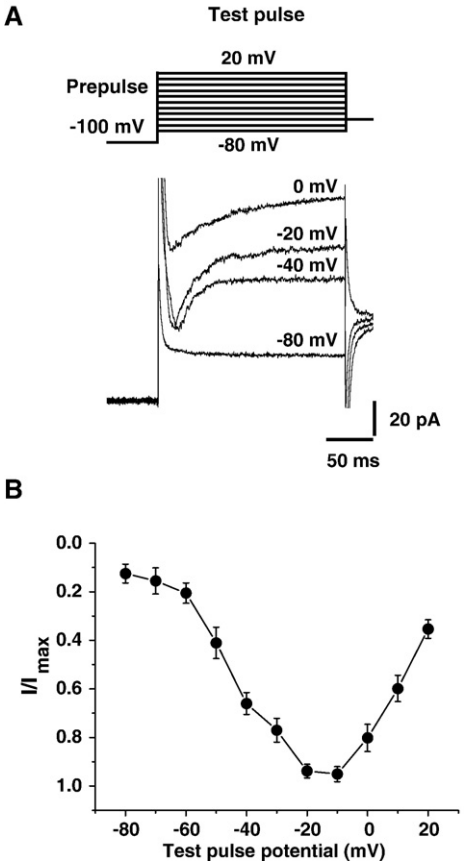

Fig. 4. Current–voltage relationship of inward currents in NCI-H295R cells. (A), Superimposed current traces of a test pulse from a typical experiment. The cell membrane was repolarized every 10 s for 1.2 s at a potential of −100 mV, then stepped for 200 ms to different potentials between 20 mV and −80 mV (10 mV steps). (B), Peak current of test pulse relative to the maximal peak current are plotted vs test pulse potentials. Data represent mean±S.E.M. of six independent experiments.

human adrenocortical NCI-H295R cells (Fig. 1A). Treatment with benidipine (3–300 nM) significantly reduced KCl- or angiotensin II-induced aldosterone production in a concentration-dependent manner (Fig. 1B). Mibefradil (1000–3000 nM), relatively a T-type selective calcium channel blocker, also inhibited aldosterone production, though with less potency than benidipine (Fig. 1B).

Since CYP11B1 and CYP11B2 play important roles in aldosterone production (Bird et al., 1995; Clyne et al., 1996; Yagci and Müller, 1996), effect of benidipine on mRNA expression level for both enzymes was investigated. KCl (10 mM) stimulation upregulated CYP11B1 and CYP11B2 mRNA expression in a time-dependent manner until 42 h. In contrast, angiotensin II (100 nM) stimulation upregulated expression only transiently with a peak of 8–12 h (Fig. 2A). Benidipine (100 or 300 nM) significantly inhibited KCl- or angiotensin II-induced mRNA expression more potently than mibefradil (3000 nM) (Fig. 2B).

KCl (1–30 mM) or angiotensin II (0.1–1000 nM) evoked Ca^{2+} influx in a concentration-dependent manner (Fig. 3A). Benidipine (10–1000 nM) and mibefradil (3000–30,000 nM) significantly inhibited KCl-evoked Ca^{2+} influx, whereas both drugs partially inhibited the angiotensin II-evoked second phase (sustained) Ca^{2+} influx (Fig. 3B).

3.2. Benidipine inhibits T-type Ca^{2+} currents in NCI-H295R cells

T-type Ca^{2+} channels in NCI-H295R cells were analyzed using standard whole cell voltage-clamp techniques. Since NCI-H295R cells express both T-type and L-type Ca^{2+} channels (Lesouhaitier et al., 2001), experimental conditions to detect T-type Ca^{2+} currents more efficiently than L-type Ca^{2+} currents were determined first. Test pulses of various potentials for 200 ms after a prepulse potential of −100 mV for 1.2 s indicated the presence of rapidly activating inward currents that were inactivated within 100–150 ms to stabilize at a sustained plateau (Fig. 4A). The amplitude of peak current as a function of voltage was maximal at −10 mV with a bell-shape characteristic of voltage-dependent Ca^{2+} currents (Fig. 4B) (Catterall et al., 2005). Inward currents were evoked by a test pulse potential of −40 mV for 200 ms, when a prepulse potential (1.2 s) was less than −60 mV (Fig. 5). These electrophysiological properties are consistent with T-type Ca^{2+} channels (Catterall et al., 2005; Perez-Reyes, 2003). Furthermore, the inhibitors of T-type Ca^{2+}

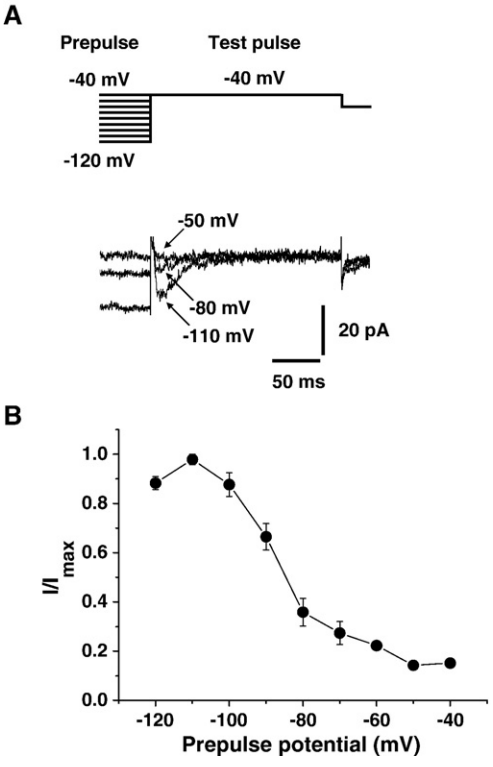

Fig. 5. Voltage-dependent recovery of inward currents from inactivation in NCI-H295R cells. (A), Superimposed current traces of a test pulse from a typical experiment. The cell membrane was repolarized every 10 s for 1.2 s at different potentials between −40 mV and −120 mV (10 mV steps), then stepped for 200 ms to −40 mV. (B), Peak current of a test pulse relative to the maximal peak current plotted vs the prepulse potential. Data represent mean±S.E.M. of five independent experiments.

channels, Ni^{2+} (100 μM) and mibefradil (1000 nM) (Fig. 6), but not the L-type specific peptidic calcium channel blocker calciseptine (De Weille et al., 1991; Teramoto et al., 1996) (data not shown), blocked the inward currents evoked by a test pulse potential of −40 mV for 200 ms after a prepulse potential of −100 mV for 1.2 s. These results indicate that the observed inward currents are attributable to T-type Ca^{2+} channels. Benidipine (10, 100 and 1000 nM) blocked the T-type Ca^{2+} currents evoked by a test pulse potential of −40 mV for 200 ms after a prepulse potential of −100 mV for 1.2 s in a concentration-dependent manner (Fig. 7). Calciseptine did not inhibit KCl- or angiotensin II-induced aldosterone production as well as T-type Ca^{2+} currents (data not shown).

3.3. Effect of other calcium channel blockers on T-type Ca^{2+} currents and aldosterone production in NCI-H295R cells

Effects of other dihydropyridine calcium channel blockers, such as efonidipine (T-type/L-type calcium channel blocker), cilnidipine (N-type/L-type calcium channel blocker) and nifedipine (L-type calcium channel blocker), were examined further. Efonidipine (100 nM) inhibited T-type Ca^{2+} currents as efficiently as benidipine (Fig. 8A). Cilnidipine (100 nM) inhibited T-type Ca^{2+} currents, however inhibitory activity was less than benidipine (Fig. 8A). Nifedipine only minimally showed any inhibitory activity (Fig. 8A). Efonidipine (3–100 nM) concentration-dependently inhibited KCl- or angiotensin II-induced aldosterone production (Fig. 8B). Cilnidipine (100 nM) reduced KCl-induced, but not angiotensin II-induced, aldosterone production (Fig. 8B). Nifedipine showed no effect on KCl- or angiotensin II-induced aldosterone production at the tested concentration (up to 100 or 300 nM) (Fig. 8B).

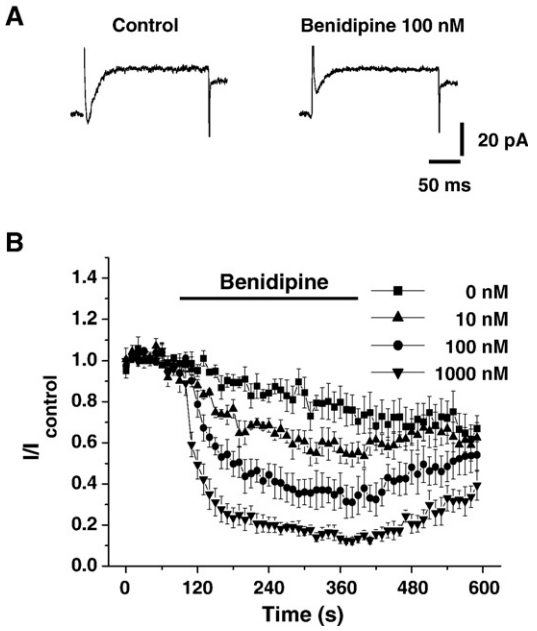

Fig. 7. Effect of benidipine on T-type Ca^{2+} currents in NCI-H295R cells. (A), Current traces of a test pulse from a typical experiment before and after application of benidipine (100 nM). The cell membrane was repolarized every 10 s for 1.2 s at a potential of −100 mV, then stepped for 200 ms to −40 mV. (B), Time course of peak currents of a test pulse relative to mean peak current of 10 test pulses before application is shown. Data represent mean±S.E.M. of five independent experiments.

3.4. Combination effects of benidipine and valsartan on aldosterone production in NCI-H295R cells

Some examples have shown the beneficial effects of combination therapy with benidipine and an angiotensin II type 1

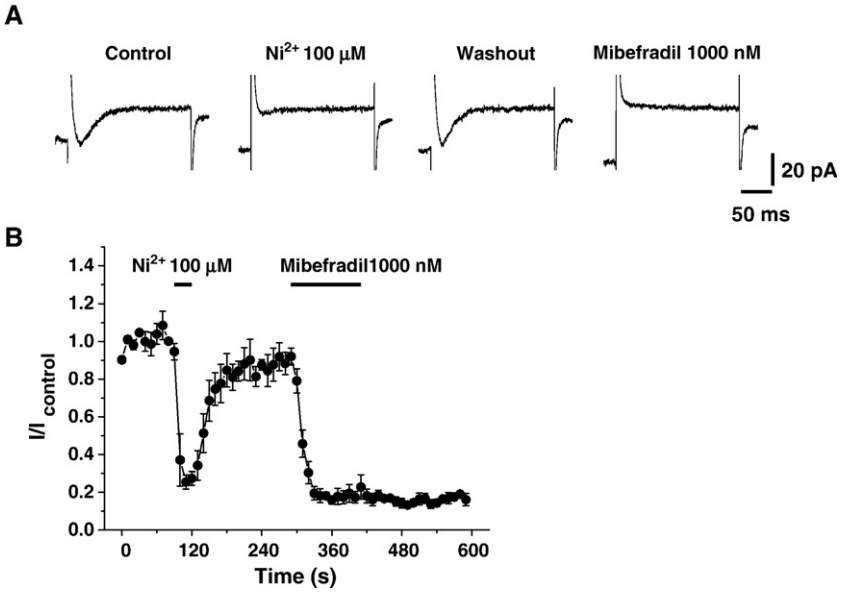

Fig. 6. Effect of $NiCl_2$ and mibefradil on T-type Ca^{2+} currents in NCI-H295R cells. (A), Current traces of a test pulse from a typical experiment before and after application of $NiCl_2$ (100 μM) or mibefradil (1000 nM). The cell membrane was repolarized every 10 s for 1.2 s at a potential of −100 mV, then stepped for 200 ms to −40 mV. (B), Time course of peak currents of a test pulse relative to mean peak current of 10 test pulses before application is shown. Data represent mean±S.E.M. of four independent experiments.

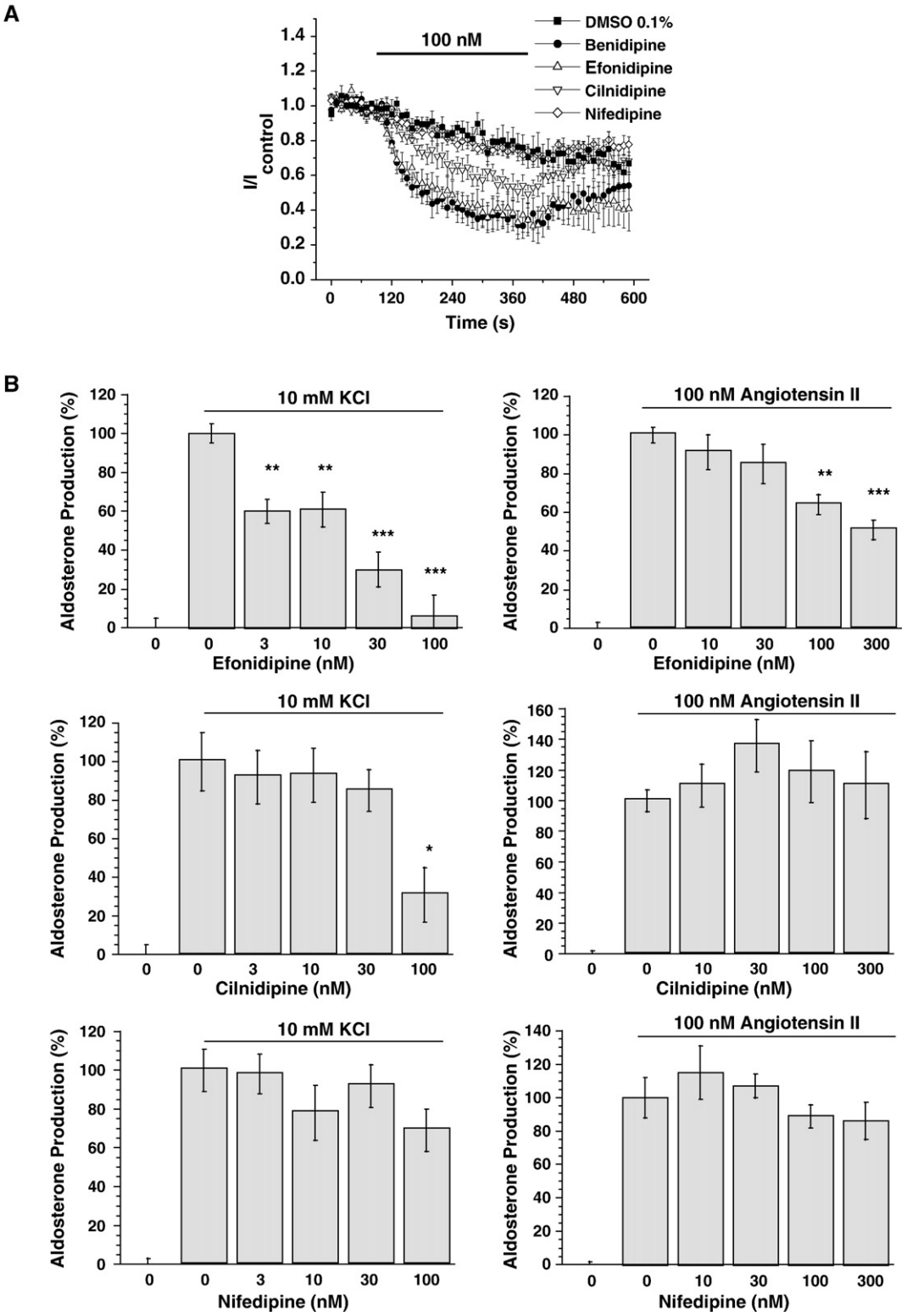

Fig. 8. Effect of other calcium channel blockers on T-type Ca^{2+} currents and aldosterone production in NCI-H295R cells. (A), The cell membrane was repolarized every 10 s for 1.2 s at a potential of −100 mV, then stepped for 200 ms to −40 mV. Time course of peak currents of a test pulse relative to mean peak current of 10 test pulses before application is shown. Data represent mean ± S.E.M. of five independent experiments. (B), NCI-H295R cells were pretreated with the indicated concentrations of efonidipine, cilnidipine or nifedipine for 10 min prior to stimulation with 10 mM KCl or 100 nM angiotensin II for 24 h. Amount of aldosterone produced in the medium was determined using ELISA and is shown as % of control. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to the control group (Dunnett test).

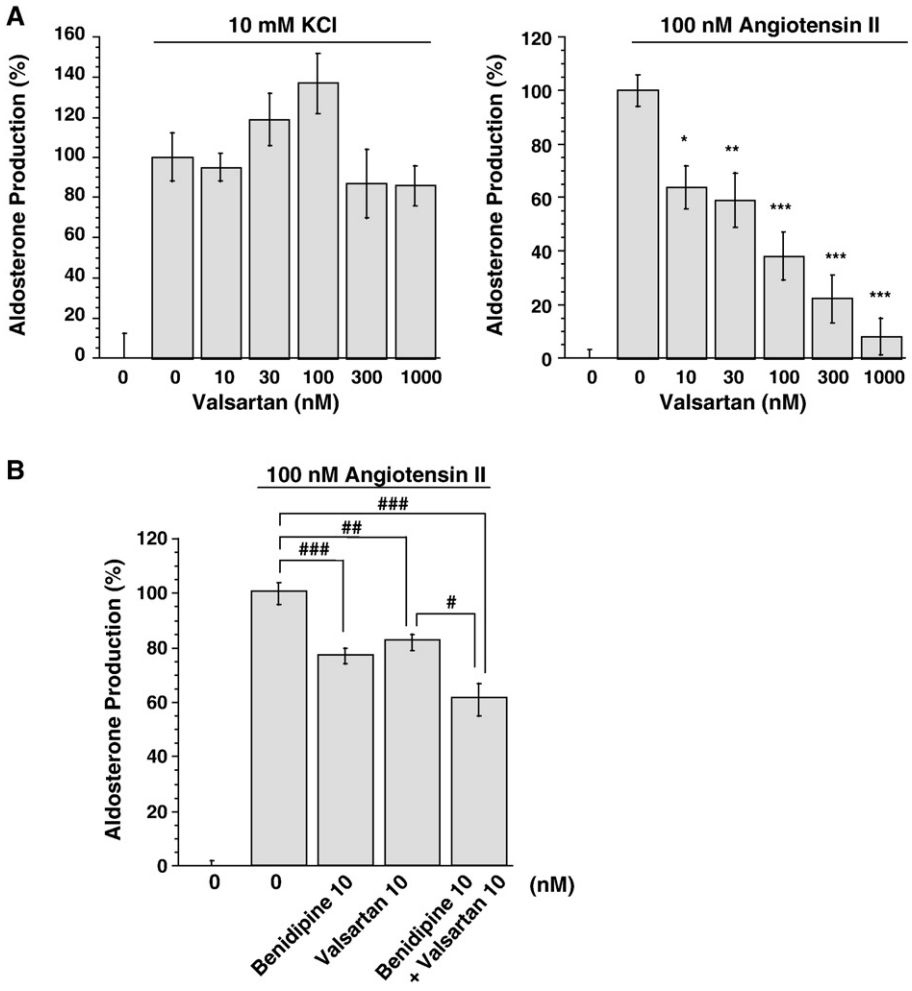

Fig. 9. Additive effect of benidipine and valsartan on aldosterone production in NCI-H295R cells. (A), NCI-H295R cells were pretreated with the indicated concentrations of valsartan for 10 min prior to stimulation with 10 mM KCl or 100 nM angiotensin II for 24 h. Amount of aldosterone produced in the medium was determined by ELISA and shown as % of control. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the control group (Dunnett test). Data represent mean±S.E.M. of six determinations in two separate experiments. (B), NCI-H295R cells were pretreated with benidipine (10 nM), valsartan (10 nM) or both at 10 min prior to stimulation with 100 nM angiotensin II for 24 h. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared to the control group. $^{\#}P<0.05$ compared to the valsartan-treated group (Student's *t*-test). Data represent mean±S.E.M. of six determinations in two separate experiments.

receptor blocker (Namba et al., 2002; Yao et al., 2003a,b). Therefore, the combination effect of benidipine and valsartan on aldosterone production in NCI-H295R cells was examined. Valsartan (10–1000 nM) significantly inhibited angiotensin II-induced, but not KCl-induced, aldosterone production (Fig. 9A). Inhibition by benidipine (10 nM) acted additively to the inhibitory effect of a lower concentration of valsartan (10 nM) on angiotensin II-induced aldosterone production (Fig. 9B).

4. Discussion

In this study, dihydropyridine calcium channel blocker benidipine was demonstrated to efficiently inhibit KCl-induced aldosterone production in human adrenocortical NCI-H295R cells at low concentrations (3 and 10 nM). In addition, benidipine was found to significantly inhibit T-type Ca^{2+} currents in NCI-H295R cells at low concentration (10 nM). Upon oral administration of 8 mg of benidipine in clinical settings, plasma concentration of benidipine reaches about 7 nM (Yao et al., 2006). Since dihydropyridine calcium channel blockers, including benidipine, are lipophilic and intracellularly accumulated (Masumoto et al., 1995), chronic administration of benidipine may give higher concentrations sufficient to inhibit aldosterone production and T-type Ca^{2+} currents in clinical settings.

Efonidipine, a dual (T-type/L-type) calcium channel blocker (Tanaka and Shigenobu, 2002), inhibited both T-type Ca^{2+} currents and KCl-induced aldosterone production as efficiently as benidipine. In contrast, L-type specific calcium channel blockers, calciseptine and nifedipine, showed no effect. As for examined calcium channel blockers, inhibitory activities for T-type Ca^{2+} currents are well correlated with aldosterone production. These results indicate that inhibition of T-type Ca^{2+} channels is responsible for the inhibition of aldosterone production in NCI-H295R cells.

NCI-H295R cells endogenously express T-type Ca^{2+} channel subtype Cav3.2 (Lesouhaitier et al., 2001), but not Cav3.1, indicating that benidipine inhibits the Cav3.2 subtype. Previous

work using *Xenopus* oocytes expressing Cav3.1 (Furukawa et al., 2005) demonstrated that benidipine inhibited Cav3.1, however, there was no information available for Cav3.2.

To elucidate the mechanism by which benidipine inhibits aldosterone production, mRNA expression for two enzymes (CYP11B1 and CYP11B2) associated with aldosterone production was examined. Expression levels of CYP11B1 and CYP11B2 mRNA were upregulated by KCl (10 mM) stimulation, and the upregulation was completely inhibited by 100 nM benidipine. Since 100 nM benidipine inhibits KCl-induced aldosterone production completely, inhibition of aldosterone production by benidipine appears to be regulated at the transcriptional level. Benidipine significantly, but not completely, inhibited KCl-evoked Ca^{2+} influx at 100 nM. These results, together with data from previous studies (Bird et al., 1995; Clyne et al., 1996; Yagci and Müller, 1996), suggest that Ca^{2+} influx is required for upregulation of CYP11B1 and CYP11B2 mRNA, however inhibition of the upregulation does not need complete inhibition of Ca^{2+} influx.

Benidipine inhibited angiotensin II-induced aldosterone production at relatively low concentrations (10 and 30 nM), however the effect was only partial even at a higher concentration (300 nM). The difference in inhibitory effects of benidipine on KCl- and angiotensin II-induced aldosterone production could be explained as follows. KCl induces membrane depolarization to activate voltage-dependent Ca^{2+} channels including T-type and L-type Ca^{2+} channels (Spat and Hunyady, 2004). In contrast, angiotensin II induces Ca^{2+} release from intracellular stores (first phase Ca^{2+} influx), followed by second phase (sustained) Ca^{2+} influx mediated by store-operated Ca^{2+} channels and voltage-dependent Ca^{2+} channels (Spat and Hunyady, 2004). In this study, benidipine inhibited KCl-evoked Ca^{2+} influx more effectively than angiotensin II-evoked Ca^{2+} influx. Benidipine did not affect angiotensin II-induced Ca^{2+} release from intracellular stores. Therefore, it seems that benidipine only inhibit Ca^{2+} influx mediated by T-type and L-type Ca^{2+} channels. In addition, angiotensin II activates signaling pathways other than Ca^{2+}-mediated pathways (Spat and Hunyady, 2004). Angiotensin II-induced upregulation of CYP11B1 and CYP11B2 mRNA is different from KCl-induced upregulation in both time course and expression level, indicating contribution from other factors besides voltage-dependent Ca^{2+} channels.

Benidipine has the unique pharmacological features of renoprotective, vascular endothelial protective and cardioprotective effects (Kitakaze et al., 1999; Yao et al., 2006). Renal protective effects of benidipine have been characterized (Yao et al., 2006). Benidipine dilates the renal efferent arterioles (Hayashi et al., 2003; Kawata et al., 1997; Yue et al., 2001) that express T-type Ca^{2+} channels (Cav3.1 and Cav3.2 subtypes) but not L-type Ca^{2+} channels (Hansen et al., 2001). Therefore, blockade of T-type Ca^{2+} channels by benidipine may be involved in the dilation of efferent arterioles (Hayashi et al., 2005). Aldosterone interacts with mineral corticoid receptors to cause end-organ damage to heart, vasculature and kidneys (Struthers and MacDonald, 2004). Aldosterone induces expression of transforming growth factor-β, fibronectin and plasminogen activator inhibitor-1 in renal glomerular mesangial cells (Lai et al., 2006; Yuan et al., 2007). In animal models, treatment with benidipine exerts renoprotective effects, such as reduction of urinary protein excretion, and inhibits transforming growth factor-β expression in the glomeruli (Nakamura et al., 2000). Inhibition of aldosterone production may also contribute to the renoprotective effects of benidipine. To clarify this possibility, further studies about effect of benidipine on plasma concentration of aldosterone are required.

Combination therapy with different types of anti-hypertensive drugs is more effective than monotherapy. Combination therapy of a dihydropyridine calcium channel blocker and an angiotensin II type I receptor blocker is recommended for treatment of hypertension. Although blockade of the renin–angiotensin system by an angiotensin II type I receptor blocker or angiotensin converting enzyme inhibitor initially reduces plasma aldosterone concentration, it returns to baseline levels with chronic use (Struthers and MacDonald, 2004). This phenomenon is referred to as "aldosterone breakthrough" or "aldosterone escape". Two recent clinical studies, the Randomized ALdosterone Evaluation Study (RALES) and the EPlerenone HEart failure and SUrvival Study (EPHESUS), have shown that mineral corticoid receptor antagonists reduce mortality in patients with congestive heart failure when used on top of angiotensin converting enzyme inhibition (Pitt et al., 1999, 2003). This effect could not be attributed solely to mineral corticoid receptor-mediated reduction of blood pressure. These clinical studies also indicate that blockade of the renin–angiotensin system does not completely inhibit aldosterone actions. Therefore, inhibition of aldosterone production by a calcium channel blocker with inhibitory activity for T-type Ca^{2+} channels, such as benidipine or efonidipine, may offer additional benefits for combination therapy with angiotensin II type I receptor blockers or angiotensin converting enzyme inhibitors, beyond their anti-hypertensive effects alone (Sato et al., 2006). Thus, an understanding of the mechanism of the existing calcium channel blockers may provide a new framework for future therapeutic strategies.

In this study, dihydropyridine calcium channel blocker benidipine was shown to efficiently inhibit both T-type Ca^{2+} currents and KCl- and angiotensin II-induced aldosterone production in human adrenocortical NCI-H295R cells at clinically available concentrations. This property of benidipine may contribute to additional benefits of this drug for treating cardiovascular and renal diseases, beyond its primary anti-hypertensive effects through the blocking of L-type Ca^{2+} channels.

Acknowledgements

The excellent technical assistance of Mr. Jun-ichi Ikeda and Ms. Maumi Ono is greatly appreciated. We are grateful to Dr. Ken Nagashima for critical reading of this manuscript and Dr. Ikufumi Yoshitake for encouragement and support.

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