Effect of T-Type Selective Calcium Antagonist on Renal Microcirculation
Studies in the Isolated Perfused Hydronephrotic Kidney

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Abstract—Although calcium antagonists exert preferential vasodilation of renal afferent arterioles, we have recently demonstrated that nilvadipine and efoniidipine, possessing both L-type and T-type calcium channel blocking action, reverse the angiotensin (Ang) II–induced afferent and efferent arteriolar constriction. In the present study, we investigated the role of T-type calcium channels in mediating the Ang II–induced efferent arteriolar tone using the selective T-type calcium channel blocker mibefradil. Isolated perfused hydronephrotic rat kidneys were used for direct visualization of renal microcirculation. Administration of Ang II (0.3 mmol/L) caused marked constriction of afferent (from 13.5±0.6 to 9.2±0.6 μm, P<0.01, n=6) and efferent (from 11.5±1.0 to 7.4±0.7 μm, P<0.01, n=5) arterioles. Mibefradil (1 mmol/L) dilated both vessels, with 82±11% and 72±7% reversal of afferent and efferent arterioles, respectively. Similarly, nickel chloride (100 mmol/L) caused dilation of both arterioles, similar in magnitude in afferent (68±10%, n=7) and efferent (80±7%, n=7) arterioles. To eliminate the possibility that the mibefradil-induced dilation was mediated by L-type channel blockade, mibefradil was administered in the presence of nifedipine (1 mmol/L). Thus, nifedipine caused modest efferent arteriolar dilation (30±6% reversal, n=9), and subsequent addition of mibefradil elicited further dilation of this vessel (80±4%, P<0.01 versus nifedipine). Furthermore, mibefradil reversed the Ang II–induced efferent arteriolar constriction even in the presence of nifedipine and phentolamine. These findings demonstrate that T-type calcium channels markedly dilate the Ang II–induced efferent arteriolar constriction, but the action is not mediated by inhibition of catecholamine release. This potent activity would contribute to the efferent arteriolar response to nilvadipine and efonidipine and may offer benefit in light of glomerular hemodynamics.

Key Words: mibefradil ■ afferent arteriole ■ efferent arteriole ■ T-type calcium channel ■ calcium antagonists ■ renal microcirculation

A growing body of evidence has accrued that the calcium antagonist, a blocker of L-type voltage-dependent calcium channels, exerts potent renal vasodilatory action in the face of systemic hypotension.1 This unique action is frequently accompanied by an elevation in glomerular filtration rate and filtration fraction.2,3 To elucidate the mechanism for these antagonists possess inhibitory action on T-type and L-type voltage-dependent calcium channels. Interestingly, α1H subunits of T-type calcium channels have been reported to exist in the efferent as well as the afferent arteriole.15 Although T-type calcium channels have been reported to play an important role in cardiac pacemaker cells12 and endocrine tissues,16 no study has examined the functional role of T-type calcium channels in vascular smooth muscle cells. Furthermore, the effect of T-type calcium channel inhibition on renal microcirculation has not yet been defined.

In the present study, we examined the role of T-type voltage-dependent calcium channels in mediating the afferent
and efferent arteriolar constriction induced by angiotensin (Ang) II, a pivotal determinant of intrarenal vascular tone, in the isolated perfused hydronephrotic kidney. To clarify these issues, we used a novel calcium antagonist, mibefradil, which possesses strong inhibitory action on T-type calcium channels but only a modest effect on L-type calcium channels.17

Methods
Chronic hydronephrosis was established in 6-week-old male Wistar rats (n=27) by the method reported previously5,7,18–21; this model facilitates direct visualization of the renal microcirculation, with responses of renal microvessels nearly parallel to those in normal kidneys.19 Furthermore, lack of tubuloglomerular feedback or systemic influence of neural and hormonal factors would allow direct assessment of renal microvascular responses.20 All procedures involving this study were conducted following the guidelines of the Animal Care Committee of Keio University.

On the day of the renal perfusion study, the rats were anesthetized with ether, and the abdominal cavity was exposed by midline incision. The hydronephrotic kidney was placed on the stage of an inverted microscope (IMT-2, Olympus) modified to accommodate a heated chamber equipped with a thin glass viewing port on the bottom surface. Kidneys were allowed to equilibrate for 30 minutes before protocols were initiated.

Kidneys were perfused with medium consisting of a Krebs-Ringer bicarbonate buffer containing 5 mmol/L d-glucose, 7.5% bovine serum albumin (Sigma), and a complement of amino acids.21 The perfusion pressure, monitored at the level of the renal artery, was maintained constant at 80 mm Hg by adjustment of the back-pressure–type regulator (10BP, Fairchild Industrial Products Co).

Vessel diameters were measured as detailed previously.5,7,18,19 Segments of afferent and efferent arterioles ~50 μm in length near the glomerulus were evaluated at 0.5- to 1.0-second intervals. To eliminate pressure-induced changes in vessel diameter, renal perfusion pressure was maintained at a constant 80 mm Hg throughout the study.

Experimental Protocols
Renal microvascular effect of mibefradil and nickel chloride (NiCl₂) was assessed under Ang II–vasoconstricted tone. After the observation of Ang II–induced (0.3 mmol/L) vasoconstrictor responses, the vasodilator effect of mibefradil (0.01, 0.1, and 1 μmol/L) and NiCl₂ (1, 10, and 100 μmol/L) on Ang II–induced vasoconstriction of afferent and efferent arterioles was assessed. Finally, losartan (1 μmol/L; Banyu Pharmaceutical Co) was added to examine whether the vasoconstrictor tone still remained after mibefradil treatment.

In additional series of experiments, the effect of mibefradil on Ang II–induced renal arteriolar constriction was assessed under L-type calcium channel blockade. After induction of Ang II (0.3 mmol/L)–induced renal vasoconstriction, nifedipine (1 μmol/L) was added to the perfusate. Thereafter, whether mibefradil (1 μmol/L) reversed the remaining constriction was examined.

It has been demonstrated that Ang II facilitated the release of catecholamines22 and mibefradil inhibited the neurotransmitter release.23 Furthermore, changes in efferent arteriolar diameter may depend on alterations in luminal pressure within this arteriole18 that ensued from afferent arteriolar dilation. To clarify whether these mechanisms contributed to the mibefradil-induced efferent arteriolar dilation, Ang II was administered in the presence of nifedipine (1 μmol/L) or nifedipine (1 μmol/L) plus phenolamine (10 μmol/L). Thereafter, the efferent arteriolar response to mibefradil (1 μmol/L) was evaluated.

Data Analysis
Data are expressed as mean±SEM. Data were analyzed by 2-way ANOVA, followed by the multiple-comparison post hoc test. A value of P<0.05 was considered statistically significant.
similar in magnitude to that in the absence of each agent (control).

NiCl₂ also dilated both afferent and efferent arterioles in a similar manner (Figure 2, upper right). Thus, 10 μmol/L NiCl₂ caused significant dilation of afferent (from 9.1±0.6 to 11.1±0.9 μm, P<0.01, n=7) and efferent (from 8.9±0.7 to 10.9±0.6 μm, P<0.01, n=6) arterioles. At 100 μmol/L, NiCl₂ restored the afferent and efferent arteriolar diameter by 68±10% (ie, to 11.5±0.7 μm) and 80±7% (to 13.0±0.6 μm), respectively.

In the next experiment, we examined the role of T-type calcium channel blocking action in mediating the mibebradil-induced efferent arteriolar dilation under the inhibition of L-type calcium channels (Figure 2, bottom). Nifedipine (1 μmol/L) elicited marked dilation of Ang II–induced afferent arteriolar constriction with almost complete restoration (ie, 86±3% reversal) of this vessel diameter (control, 13.9±0.7 μm; Ang II, 8.0±0.6 μm; nifedipine, 13.2±0.7 μm; n=7). In striking contrast, the Ang II–induced efferent arteriolar constriction (from 13.0±0.5 to 7.9±0.4 μm, P<0.01, n=9) was less responsive to nifedipine, with 30±6% dilation of this arteriole (to 9.5±0.3 μm, P>0.05). Subsequent addition of mibebradil further increased the efferent arteriolar diameter to 11.9±0.4 μm (P<0.01, n=9), corresponding to 80±4% reversal of the Ang II–induced constriction. In the absence of vasoactive stimuli, however, neither nifedipine nor mibebradil had an effect on basal diameter of afferent and efferent arterioles (data not shown).

Finally, whether mibebradil reversed the Ang II–induced efferent arteriolar constrictor tone was assessed in the presence of nifedipine or nifedipine plus phentolamine (Figure 3). Thus, Ang II had no effect on afferent arteriolar tone in the presence of nifedipine (P>0.5, n=7) and nifedipine plus phentolamine (P>0.5). In the efferent arteriole, Ang II induced 25±9% and 21±1% vasoconstriction in the presence of nifedipine (P<0.01, n=9) and nifedipine plus phentolamine (P<0.01, n=4), respectively; these decrements did not differ from those in the absence of these agents (ie, control, 33±3% decrements, P>0.5). Further addition of mibebradil markedly inhibited the Ang II–induced efferent arteriolar constriction, with 69±6% reversal in the presence of nifedipine, and 74±18% reversal in the presence of nifedipine plus phentolamine.

**Discussion**

It is well established that calcium antagonists possess potent vasodilator activity and are widely used as antihypertensive and coronary vasodilator agents. Renal vascular beds are also primary targets for the calcium antagonist–induced vasodilator action. Indeed, calcium antagonists are reported to relax renal vascular beds before the systemic blood pressure is reduced. Such divergent activity of the calcium antagonist is also observed within the renal vasculature. Thus, the calcium antagonist predominantly dilates preglomerular vessels, including afferent arterioles, whereas efferent arterioles are relatively refractory to the vasorelaxing action of this agent. These characteristics are demonstrated to be attributed to the preferential distribution of L-type calcium channels in afferent but not efferent arterioles and are closely associated with the renal hemodynamic change, ie, elevated filtration fraction. It has been recognized, however, that some calcium antagonists, including efonidipine and nilvadipine, manifest decreases or no changes in filtration fraction. In this regard, these calcium antagonists possess the T-type and L-type calcium channel blocking activity. Nevertheless, no investigations have been conducted examining the role of T-type calcium channels in modifying the efferent arteriolar tone.

The present study has demonstrated that mibebradil reverses the Ang II–induced constriction of both afferent and efferent arterioles; the ability of this blocker to inhibit the Ang II–induced constriction is nearly the same in these arterioles. In this regard, Nakamura et al. studied the renal micropuncture technique, have recently observed similar findings that mibebradil reduces both afferent and efferent arteriolar resistance, although the change in afferent arteriolar resistance is greater. Speculatively, this unique action of mibebradil is associated with the blocking action of T-type calcium channels, because we have recently demonstrated that nilvadipine and efonidipine, both of which possess the inhibitory activity on T-type calcium channels, relax efferent and afferent arterioles (Figure 4). Indeed, in the present study, we have found that NiCl₂ also dilates both arterioles. Taken together, the unique action of these calcium antagonists, sharing T-type calcium channel blocking activity, would be anticipated to produce renal arteriolar action by modifying T-type calcium channels. Of note, in the presence of nifedipine, mibebradil causes no further dilation of the afferent arteriole but potently reverses the Ang II–induced efferent arteriolar constriction. Similarly, pretreatment with nifedipine, through which the Ang II–induced afferent arteriolar constriction was prevented, did not alter the mibebradil-induced efferent arteriolar response (Figure 3). These findings would eliminate the possibility that the elevated
channel blockers. Similarly, Hermsmeyer and Miyagawa30 observed that the endothelin-induced vascular contraction was inhibited by mibebradil but not amloidipine. Thus, the receptor-mediated sustained vasoconstriction, which activates intracellular vasoconstrictor mechanisms (protein kinase-C and inositol trisphosphate pathways), is linked to T-type calcium channels, but the mode of contribution of T-type channels to arteriolar constriction may differ greatly from that in depolarization (eg, KCl)-stimulated T-type channel stimulation. Finally, mibebradil is recently suggested to activate K\textsubscript{ATP} channels,33 which could dilate effenter arterioles.34

The present study demonstrates that both mibebradil and NiCl\textsubscript{2} potently inhibit the Ang II–induced effenter arteriolar constriction (Figure 2). On the basis of the pharmacological property of these agents, this effect most likely is mediated by the L-type calcium channel blocking action. Alternatively, these agents also inhibit T-type calcium channels and subsequently could dilate effenter arterioles. Of note, nifedipine completely dilates this vessel, and no additive effect is obtained with mibebradil (Figure 2, bottom). Provided that both L-type and T-type calcium channels coexist functionally in the effenter arteriole, there should be some overlap in L-type- and T-type calcium channel–mediated vasoconstrictor mechanisms; T-type channel activation is reported to facilitate Ca\textsuperscript{2+} release from sarcoplasmic reticulum in cardiac myocytes35 and affects the protein kinase C–mediated pathway and the vascular smooth muscle contraction,30 both of which constitute intermediate components between Ang II receptor activation and L-type calcium channel opening.31,32 Although this conjecture appears intriguing, it remains a matter of controversy whether T-type calcium channels operate in the effenter arteriole.15

In contrast to well-established roles of L-type calcium channels, functions of T-type calcium channels within the renal vasculature remain fully undetermined. It is generally accepted that T-type calcium channels are involved in the spontaneous firing of calcium-dependent action potential and vasomotion.12 Although the relation between these mechanisms and the actual contribution to vascular tone remains undetermined, our present observations and results from other laboratories26 clearly demonstrate a substantial role of T-type calcium channels in the control of renal microvascular tone. These vasoactive effects of T-type calcium channel antagonists would influence the development of renal injury. Thus, several lines of recent studies have reported that mibebradil ameliorates the progression of renal injury in a variety of hypertensive renal injury models, including desoxycorticosterone acetate salt hypertensive rats36 and spontaneously hypertensive rats.26 Of note, some novel calcium antagonists with vasodilator action on both afferent and effenter arterioles, including efonidipine and nilvadipine, are demonstrated to retard the progression of chronic renal injury.37,38 As expected from the renal microvascular action, therefore, the amelioration of renal injury by mibebradil may be attributable to the potent effenter arteriolar vasodilator action of this agent. Nevertheless, mibebradil is also reported to possess several other actions, such as antiproliferative effects.39 To determine whether mibebradil-induced amelioration in renal injury is related to renal microcirculatory action requires further investigations.

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**Figure 4.** Heterogeneity in renal vasodilator action of calcium antagonists. Nifedipine (1 \textmu mol/L), nilvadipine (1 \textmu mol/L), mibebradil (1 \textmu mol/L), NiCl\textsubscript{2} (100 \textmu mol/L), and efonidipine (1 \textmu mol/L) were administered during Ang II–induced afferent and effenter arteriolar constriction, and the ratio of effenter/afferent arteriolar constriction was compared. *P<0.05 vs nifedipine; †P<0.05 vs nilvadipine. Some of the data are modified from Hayashi et al5 and Ozawa et al.10

glomerular pressure that follows the effenter arteriolar relaxation passively dilates the effenter arteriole but would rather reflect active vasodilation of this vessel.

It has been demonstrated that mibebradil possesses inhibitory action on L-type calcium channels, although this activity is less than that on T-type calcium channels.17 In the effenter arteriole, however, L-type calcium channels appear sparse because effenter arterioles are refractory to the vasodilator action of the calcium antagonist4–6 or the vasoconstrictor action of BAY K-8644, an L-type calcium agonist.27 Furthermore, the present study shows that during the blockade of L-type calcium channels by nifedipine, mibebradil retains the ability to dilate the effenter arteriole. Collectively, these observations provide strong evidence for the mechanism of mibebradil-induced effenter arteriolar dilation that is independent of L-type calcium channel blockade but rather is associated with the inhibition of T-type calcium channel–mediated mechanisms.

Although the present study suggests the involvement of T-type calcium channels in Ang II–induced renal microvascular tone, the mechanisms for the T-type calcium channel–mediated vasoconstriction remain undetermined. It is well known that Ang II facilitates the release of catecholamines in the nerve terminal.22 Thus, the effenter arteriolar action of mibebradil, which also possesses N-type calcium channel blocking activity,28 might be mediated in part by the inhibition of Ang II–induced norepinephrine release within the effenter arteriole.23 The present study, however, militates against this possibility because pretreatment with phenolamine does not alter the mibebradil-induced dilation of the effenter arteriole (Figure 3). Alternatively, T-type calcium channels may be activated through protein kinase C–mediated pathways,29,30 an important mechanism for the effenter arteriolar tone during Ang II constriction.31,32 Thus, unlike the relatively pure stimulation of T-type calcium channels by depolarization, which causes transient increases in calcium entry, Ang II elicits the sustained effenter arteriolar constriction, which is inhibited by a variety of T-type but not L-type calcium channel blockers. Similarly, Hermensmeyer and Miyagawa30 observed...
In summary, the present study demonstrates that mibe-
fradil, with predominant blocking activity on T-type calcium
channels, reverses both afferent and efferent arteriolar vaso-
constriction induced by Ang II. Furthermore, NiCl₂, sharing
the same property with mibebradil, exerts similar microvas-
cular action, suggesting an important contribution of T-type
calcium channels in the regulation of efferent arteriolar tone.
In concert with predominant activity of L-type calcium
channels, the present study clearly demonstrates the hetero-
geneity in the subtype of calcium channels within the renal
microvasculature, and such differences may determine the
reactivity of the renal vasculature to calcium antagonists.

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