British Journal of Pharmacology (1998) 124, 579–585
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T-type and L-type calcium channel blockers exert opposite effects on renin secretion and renin gene expression in conscious rats

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1 This study aimed to investigate and to compare the effects of pharmacological T-type calcium channel and of L-type calcium channel blockade on the renin system. To this end, male healthy Sprague-Dawley rats were treated with the T-channel blocker mibefradil or with the L-channel blocker amlodipine at doses of 5 mg kg^{-1}, 15 mg kg^{-1} and 45 mg kg^{-1} per day for four days and their effects on plasma renin activity (PRA) and kidney renin mRNA levels were determined.

2 Whilst amlodipine lowered basal systolic blood pressure at 5 mg kg^{-1}, mibefradil had no effect on basal blood pressure in the whole dose range examined. Amlodipine dose-dependently induced up to 7 fold elevation of PRA and renin mRNA levels. Mibefradil significantly lowered PRA and renin mRNA levels at 5 mg kg^{-1} and moderately increased both parameters at a dose of 45 mg kg^{-1}, when PRA and renin mRNA levels were increased by 100% and 30%, respectively. In primary cultures of renal juxtaglomerular cells neither amlodipine nor mibefradil (0.1–10 μM) changed renin secretion.

3 In rats unilateral renal artery clips (2K-1C) mibefradil and amlodipine at doses of 15 mg kg^{-1} day^{-1} were equally effective in lowering blood pressure. In contrast mibefradil (5 mg kg^{-1} and 15 mg kg^{-1} day^{-1}) significantly attenuated the rise of PRA and renin mRNA levels, whilst amlodipine (15 mg kg^{-1}) additionally elevated the rise of PRA and renin mRNA levels in response to renal artery clipping.

4 These findings suggest that T-type calcium channel blockers can inhibit renin secretion and renin gene expression *in vivo*, whilst L-type calcium channel blockers act as stimulators of the renin system. Since the inhibitory effect of T-type antagonists is apparent *in vivo* but not *in vitro*, one may infer that the effect on the renin system is indirect rather than directly mediated at the level of renal juxtaglomerular cells.

Keywords: Mibefradil; amlodipine; plasma renin activity; renin mRNA

Introduction

Calcium ions play a pivotal role in the contraction of vascular smooth muscle cells and consequently in vascular resistance. Calcium antagonists, in particular inhibitors of transmembrane calcium entry into vascular smooth muscle cells (VSMC), are therefore powerful drugs in the treatment of hypertension (Cauvin *et al*., 1983; Hermsmeyer, 1991; Burges & Moisey, 1994). The most effective in this context have been proven to be inhibitors of L-type calcium channels (MacCarthy, 1987; Osswald *et al*., 1990). A major side effect of L-type calcium antagonists in the treatment of hypertension is activation of the renin-angiotensin system (Churchill, 1987; Osswald *et al*., 1990; Cappucio *et al*., 1991; Schricker *et al*., 1996a) which may, at least partly, counteract the hypotensive effect of calcium antagonists. The stimulation of the renin system by calcium channel antagonists is probably a multifactorial effect involving systemic effects such as a fall in blood pressure (Messing *et al*., 1991; Bond & Boot, 1992) and activation of sympathetic outflow (Kailasam *et al*., 1995) on the one hand, and more direct effects on the level of renal juxtaglomerular (JG) cells, which are the main site of renin gene expression and secretion. From these cells, renin is secreted by regulated exocytosis and evidence has been accumulated to indicate that exocytosis of renin is inhibited by a rise in the cytosolic calcium concentration (Hackenthal *et al*., 1990).

Impairment of calcium entry into JG cells, which are metaplastically transformed vascular smooth muscle cells, is therefore expected to stimulate the secretion of renin. The pathways by which calcium enters renal renin-producing cells, have not clearly been identified but may involve store-regulated calcium influx directly in the juxtaglomerular region and L-type calcium channels in the more proximal parts of renal afferent arterioles (Scholz & Kurtz, 1996). Both pathways are inhibited by the second generation of dihydropyridines (Kass *et al*., 1991), such as amlodipine.

Recently, a new generation of calcium channel blockers has been developed including mibefradil (Clozel *et al*., 1990; Veniant *et al*., 1991a,b) which selectively blocks T-type calcium channels (Mishira & Hermsmeyer, 1994). Mibefradil has been demonstrated to have similar potency to L-type calcium channels blockers in lowering blood pressure during hypertensive states (Hefti *et al*., 1990; Veniant *et al*., 1993; 1994). How T-type channel blockers influence the renin system, in particular renin secretion and renin gene expression, is not yet known.

We are interested therefore to compare the effects of an established L-type channel blocker, such as amlodipine, with a T-type channel blocker, such as mibefradil, on the renin system in conscious animals as well as in isolated juxtaglomerular cells. Our findings show that low dose mibefradil inhibits renin secretion and renin gene expression *in vivo* but not *in vitro*. At higher doses mibefradil induced a moderate stimulation of renin secretion and renin gene expression. However, this was substantially smaller than the respective stimulation induced by amlodipine.

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Methods

Animal experiments

Male Sprague-Dawley rats (220–250 g) kept in the local animal house were used for these studies. For blockade of calcium channels, the animals were treated either with the L-type calcium channel antagonist amlodipine or with the T-type calcium channel antagonist mibefradil (Ro 40-5967, Hoffmann-La Roche, Basel, Switzerland) at daily doses of 5 mg kg^{-1}, 15 mg kg^{-1} and 45 mg kg^{-1} for four days. The drugs were applied by gavage in the morning of each experimental day. In the control group, water was applied by gavage at the same time points.

In a second series of experiments, amlodipine (15 mg kg^{-1} day^{-1}) or mibefradil (5 mg kg^{-1} and 15 mg kg^{-1} day^{-1}) were given for four days to rats carrying a left renal artery clip from the third day of drug treatment. For this purpose animals were anaesthetized with methohexitone (50 mg kg^{-1}) and then the left kidney was exposed by an abdominal incision, and sterile silver clips (Degussa AG, Darmstadt, Germany) with an inner diameter of 0.2 mm were placed on the left renal arteries. The corresponding control group to this set of experiments consisted of rats also carrying a left renal artery clip but not treated with amlodipine or mibefradil. Animals were examined two days after insertion of the renal artery clips.

At the end of the experiment the animals were killed by decapitation and blood was collected from the carotid arteries, EDTA (5 mM) was added to the blood and haematocrit and PRA were determined. The kidneys were rapidly removed, weighed, cut into halves and rapidly frozen in liquid nitrogen. The organs were stored at −80°C until isolation of total RNA, which was extracted from one of the frozen halves of kidneys as described by Chomczynski & Sacchi (1987).

Determination of preprorenin mRNA by RNase protection assay

Renin mRNA was measured by RNase protection as described previously (Holmer *et al*., 1994). A preprorenin cRNA probe containing 296 base pairs of exon I and III, generated from a pGEM-4 vector carrying a PstI-KpnI restriction fragment of a rat preprorenin cDNA (Burnham *et al*., 1987) was generated by transcription with SP6 RNA polymerase (Amersham Int., Amersham, U.K.). Transcripts were routinely labelled with [^{32}P]-GTP (400 Ci $mmol^{-1}$; Amersham International) and purified on a Sephadex G50 spin column. For hybridization total kidney RNA was dissolved in a buffer containing 80% v/v formamide, 40 mM piperazine-N,N′-*bis*(2-ethane sulphonic acid), 400 mM NaCl, 1 mM EDTA (pH 8); 20 μg of kidney RNA were hybridized in a total volume of 50 μl at 60°C for 12 h with 5×10^5 c.p.m. radiolabelled renin probe. RNase digestion with RNase A and T1 was carried out at room temperature for 30 min and terminated by incubation with proteinase K (0.1 mg ml^{-1}) and SDS (0.4% w/v) at 37°C for 30 min.

Protected preprorenin mRNA fragments were purified by phenol/chloroform extraction, ethanol precipitation and subsequent electrophoresis on a denaturing 8% w/v polyacrylamide gel. After autoradiography of the dried gel at −80°C for one day bands representing protected renin mRNA fragments were excised from the gel and radioactivity was counted with a liquid scintillation counter (1500 Tri-CarbTm, Packard Instrument Company, Downers Grove, Illinois, U.S.A.).

Determination of glyceraldehyde-3-phosphate dehydrogenase-mRNA (GAPDH)

The presence of rat GAPDH-mRNA in total RNA was measured by RNase protection assay. A GAPDH-cRNA probe containing a fragment of 342 bp of rat GAPDH-cDNA (Tso *et al*., 1985) was generated from a pGEM-4Z vector (Pharmacia) after linearization with *Hind*III and following transcription with SP6-polymerase. GAPDH-mRNA was used as a standard RNA controlling the quality of the RNA preparation. Total RNA (1 μg) was hybridized under the conditions described for the determination of preprorenin mRNA.

Measurement of heart rate and blood pressure

Heart rate and systolic blood pressure was measured by the tail cuff method with a BP recorder 8005 (Rhema, Hofheim, Germany) at 08 h 00 min and 16 h 00 min of each experimental day.

Determination of plasma renin activity

PRA was determined by incubation of the plasma samples at 37°C (pH 6) for 1 h and measurement of the angiotensin I, generated using a commercially available radioimmunoassay kit for angiotensin I (Sorin Biomedica, Düsseldorf, Germany).

Experiments with cultured juxtaglomerular cells

Mouse juxtaglomerular cells were isolated as described previously (Della Bruna *et al*., 1992). In order to prepare 3 ml of final cell suspension, one C57BL6 mouse (4–6 weeks old) that had free access to normal food and water (Altromin, Lage, Germany) was killed by decapitation. The kidneys were removed, decapsulated and minced with a scalpel blade. The minced tissue was incubated with gentle stirring in buffer 1 (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM glucose, 20 mM sucrose, 10 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.4) supplemented with 0.25% w/v trypsin (Sigma, Deisenhofen, Germany) and 0.1% collagenase (0.5 u mg^{-1}, type A, Boehringer Mannheim, Germany) at 37°C for 70 min. After enzymatic dissociation, the tissue was sieved over a 22 μm screen. Single cells passing the screen were collected, washed and resuspended in 4 ml of buffer 1 and then further separated using Percoll (Pharmacia, Uppsala, Sweden) density gradients. The cell suspension obtained was added to two tubes each containing 30 ml of 30% v/v isoosmotic Percoll in buffer 1. After 25 min of centrifugation at 4°C and 27,000 *g*, four cell layers with different specific renin activity were obtained. The cellular layer (density = 1.07 g ml^{-1}) with the highest specific renin activity was used for cell culture.

These cells were washed in buffer 1 and resuspended in RPMI-1640 medium (Biochrom, Berlin, Germany) containing 0.66 u ml^{-1} insulin, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2% v/v foetal calf serum (FCS). The cultures were distributed in 100 μl aliquots into 96-well plates. The cultures were incubated at 37°C in humidified atmosphere containing 5% CO_2 in air.

After 24 h of primary culture, the culture medium was removed, and the cultures were washed once with 100 μl RPMI-1640 medium containing 2% v/v FCS. Then 100 μl of fresh and prewarmed culture medium with the chemicals to be tested were added.

Experiments on renin secretion were performed for the next 20 h of incubation. At the end of experiments,

supernatants were collected and centrifuged at 1000 g at room temperature to remove cellular debris. The supernatants were then stored at −20°C until assayed for renin activity. Cells were lysed by adding to each culture well of 100 μl PBS containing 0.1% Triton X-100 and shaking for 45 min at room temperature. The lysed cells were stored at −20°C until further processing.

Renin secretion rates were estimated from the appearance rate of renin in the culture medium. To minimize differences between different cell culture preparations, renin secretion rates were calculated as fractional release of total renin found at the end of primary culture (i.e. renin activity released/(renin activity released + renin activity remaining in the cells)).

Statistics

Significance levels were calculated with the ANOVA test. A value of $P<0.05$ was considered significant.

Results

Effect of drugs on systolic blood pressure

Animals were treated with the drugs for 4 days. At that time systolic blood pressure was significantly lowered in animals receiving amlodipine but not in animals receiving mibefradil (Figure 1). The lowest dose of amlodipine used in this study (5 mg kg^{-1} day^{-1}) lowered systolic blood pressure by about 20 mmHg.

Effect of drugs on heart rate

The lowest dose of amlodipine used (5 mg kg^{-1} day^{-1}) increased heart rate by about 20 beats min^{-1} (Figure 2). The increase in heart rate was not further enhanced by higher doses of amlodipine but the scatter of values became smaller with high doses of amlodipine. In contrast, mibefradil exerted a clear dose-dependent inhibitory effect on heart rate (Figure 2).

Effect of drugs on plasma renin activity

PRA was determined in the animals as an indirect measure of renal renin secretion. Figure 3 shows that amlodipine caused a dose-dependent rise of PRA which was elevated by 500% above normal in animals treated with the highest dose of 45 mg kg^{-1} day^{-1}. Mibefradil treatment exerted a dual effect on PRA values. At a dose of 5 mg kg^{-1} day^{-1}, PRA was significantly lowered. At a dose of 15 mg kg^{-1} day^{-1} PRA values were not different from controls. At 45 mg kg^{-1} day^{-1} mibefradil PRA values were significantly increased by about 200% (Figure 3b).

Effect of drugs on renal renin mRNA levels

Similar to its effect on PRA, amlodipine also dose-dependently increased renin mRNA levels by about 700% with the highest dose of 45 mg kg^{-1} day^{-1} (Figure 4a). Mibefradil at a dose of 5 mg kg^{-1} day^{-1} moderately but significantly lowered renin mRNA levels. At the highest dose of 45 mg kg^{-1} day^{-1}, renin mRNA levels were increased by about 30% versus control animals without reaching a level of statistical significance ($P>0.05$) (Figure 4b).

Effects of drugs on renin secretion from isolated juxtaglomerular cells

To test for a possible direct effect of amlodipine and of mibefradil at the level of the renal juxtaglomerular cells, the effects of both drugs were examined in primary cultures of mouse juxtaglomerular cells. As shown in Figure 5 neither drug affected basal renin secretion from cultured cells, which

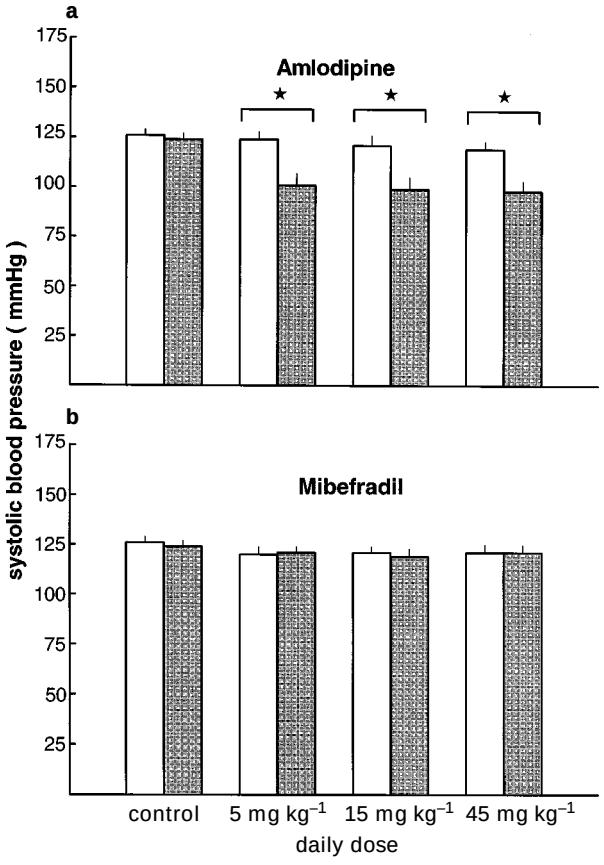

Figure 1 Dose-response effect of treatment with amlodipine (a) and mibefradil (b) for 4 days on basal systolic blood presure in conscious rats. Data show values of systolic blood pressure measurements (mmHg) at the beginning (open columns) and at the end (solid columns) of the experiments. Data are mean ± s.e.mean, $n=6$; $^*P<0.05$.

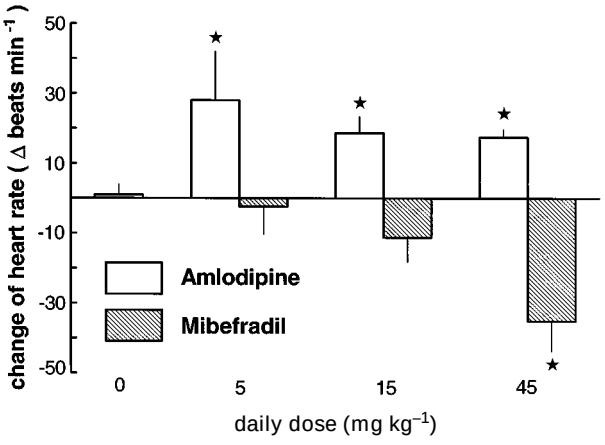

Figure 2 Dose-response effect of treatment with amlodipine and mibefradil for 4 days on heart rate in the conscious rat. Data show changes in heart rate (beats min^{-1}) and are means ± s.e.mean, $n=6$; $^*P<0.05$.

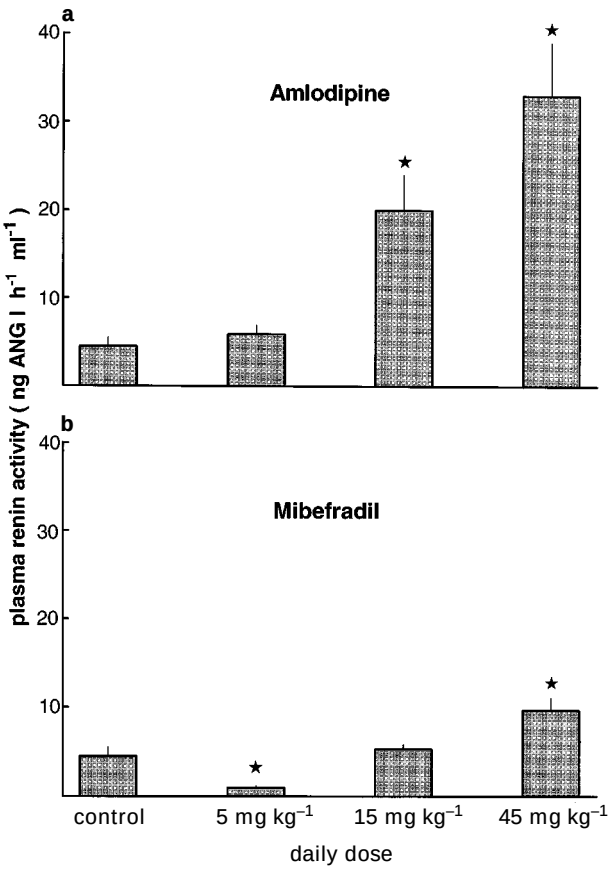

Figure 3 Dose-response effect of treatment with amlodipine (a) and mibefradil (b) for 4 days on PRA. Data show changes in PRA (ng angiotensin I h^{-1} ml^{-1}) and are means ± s.e.mean, $n=6$; $^*P<0.05$.

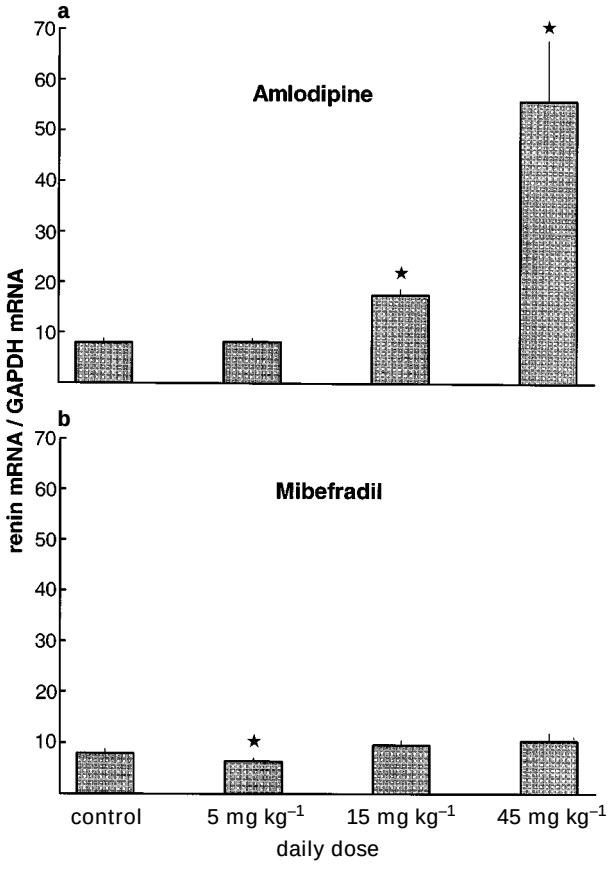

Figure 4 Dose-response effect of treatment with amlodipine (a) and mibefradil (b) for 4 days on renin mRNA. Data show changes in renal renin mRNA levels and are means ± s.e.mean, $n=6$, $^*P<0.05$.

spontaneously released about 18% of stored active renin within 20 h of culture. Renin secretion was significantly stimulated by a lowering of the extracellular calcium concentration and by activation of adenylate cyclase with forskolin (Figure 5).

Effect of drugs on plasma renin activity, renin mRNA levels and blood pressure in 2K-1C rats

To examine the effects of calcium antagonists on the stimulated renin system, amlodipine and mibefradil were administered to rats with unilateral renal artery stenosis. In animals without drug treatment, PRA increased about four fold two days after clipping (Figure 6). At that time, renin mRNA was increased about five fold in the clipped kidneys and had decreased to about 30% of the normal value in the intact contralateral kidney (Figure 7). In animals pretreated with amlodipine (15 mg kg^{-1} day^{-1}) PRA increased about 10 fold two days after clipping (Figure 6). Renin mRNA in the clipped kidney had increased about six fold above control (Figure 7a). Renin mRNA levels in the contralateral intact kidney were not different from the values found in normal untreated animals (Figure 7b). In animals pretreated with mibefradil (5 mg kg^{-1} day^{-1}) the increase in PRA, as well as the increase in renin mRNA in the stenosed kidneys two days after clipping, were greatly reduced (Figure 7a), whilst renin mRNA in the contralateral intact kidney fell significantly below the value found in normal kidneys (Figure 7b). At a dose of 15 mg kg^{-1} day^{-1} mibefradil still attenuated the

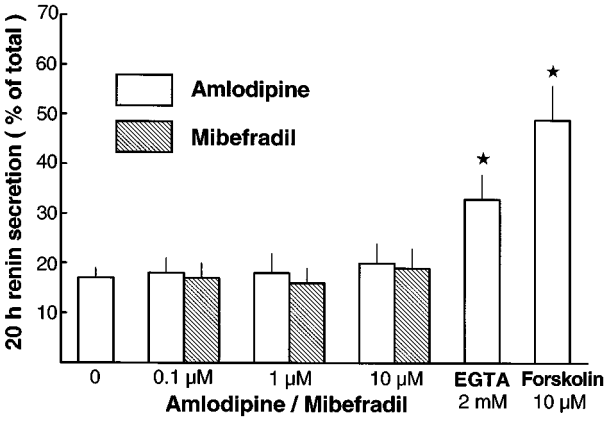

Figure 5 Renin secretion from mouse cultured JG cells under basal conditions (control) and in the presence of amlodipine (0.1 – 10 μM), mibefradil (0.1 – 10 μM), EGTA (2 mM) and forskolin (10 μM). Data are means ± s.e.mean of different cell preparations, $n=5$. Total renin activity in the different preparations was 48 ± 12 μg angiotensin I h^{-1} mg^{-1} cellular protein. Each experiment represents the mean of 4 replicate culture wells. $^*P<0.05$.

increase of PRA and of renin mRNA levels in response to renal artery clipping.

Unilateral renal artery clipping increased systolic blood pressure by about 30 mmHg (Figure 8). In animals receiving either amlodipine or mibefradil (15 mg kg^{-1} day^{-1}) together

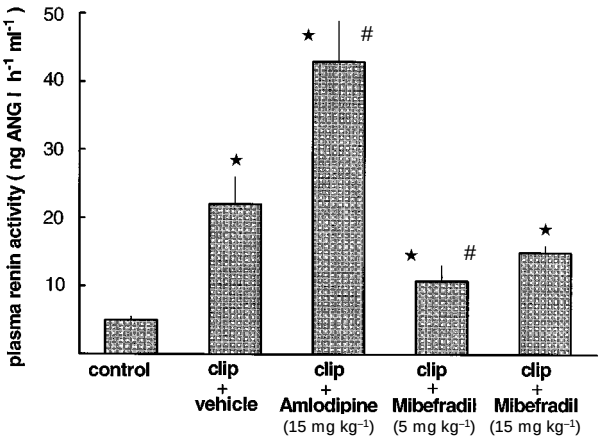

Figure 6 Effect of treatment with amlodipine and mibefradil for 4 days on PRA in 2K-1C rats. Data show changes in PRA (ng angiotensin h^{-1} mg^{-1}) and are means ± s.e.mean, $n=6$; $^*P<0.05$ vs control; $\#P<0.05$ vs clipped, vehicle-treated animals.

with a renal artery clip, systolic blood pressure was not changed relative to the starting values (Figure 8). At a dose of 5 mg kg^{-1} day^{-1} mibefradil did not prevent the increase in blood pressure in response to renal artery clipping (Figure 8).

Discussion

This study aimed to compare the effects of the L-type calcium channel blocker amlodipine and the T-type calcium channel blocker mibefradil on renin secretion and renin gene expression. In accordance with previous results (Schricker *et al*., 1996a) we found that amlodipine in the concentration range between 5 mg kg^{-1} day^{-1} and 45 mg kg^{-1} day^{-1} lowered systolic blood pressure at the lowest dose examined in this study. Mibefradil on the other hand exerted no effect on basal systolic blood pressure over the whole dose range examined. The inability of mibefradil to lower basal blood pressure seems to be at odds with previous studies showing a reduction of blood pressure in normal animals (Hefti *et al*., 1990). The majority of these studies used i.v. bolus injections of mibefradil and the experimental protocol is therefore not comparable with this study (e.g. Veniant *et al*., 1991a). One study showed that mibefradil administered by daily gavage significantly lowered systolic blood pressure after six days of treatment (Schmitt *et al*., 1995). However, in that study basal blood pressure of rats was substantially higher than in the present experiments. Thus mibefradil lowered systolic blood pressure to values between 125 to 130 mmHg which were considered as normal blood pressure values in this study, whilst amlodipine lowered blood pressure to values around 100 mmHg what was also found in this study. It appears that orally administered mibefradil has little effect on normal blood pressure values and is primarily effective during hypertensive states (Boulanger *et al*., 1994). This conclusion is supported by the observation that mibefradil prevented the rise of blood pressure in response to renal artery clipping.

In contrast to its lack of effect on basal blood pressure, mibefradil dose-dependently lowered the heart rate, which is in good agreement with previous observations (Veniant *et al*., 1991a). The mechanism of the negative chronotropic effect of mibefradil is not yet understood but could involve direct effects on the sinoatrial node as well as indirect effects such as a decrease in sympathetic activity. In contrast to mibefradil and

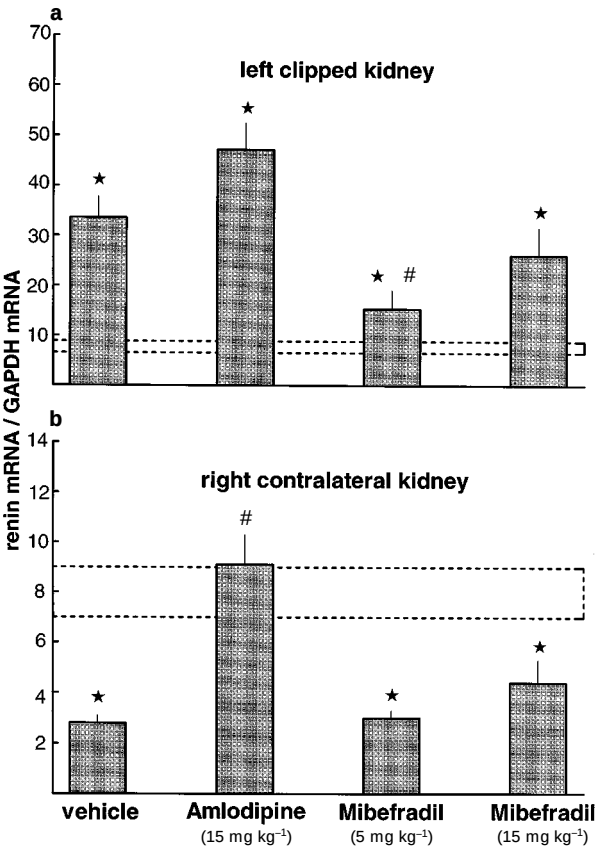

Figure 7 Effect of treatment with amlodipine and mibefradil for 4 days on renin mRNA in clipped (a) and contralateral (b) kidneys of 2K-1C rats. Data show changes in renal renin mRNA levels and are means ± s.e.mean, $n=6$; $^*P<0.05$ vs control; $\#P<0.05$ vs clipped, vehicle-treated animals.

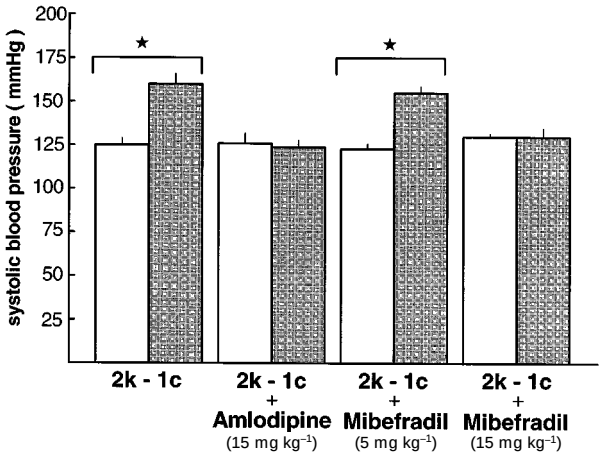

Figure 8 Effect of treatment with amlodipine and of mibefradil for 4 days on systolic blood pressure in conscious 2K-1C rats. Data show values of blood pressure measurements (mmHg) at the beginning (open columns) and at the end (solid columns) of the experiments. Data are means ± s.e.mean, $n=6$; $^*P<0.05$.

in accordance with previous observations (Veniant *et al*., 1991a), amlodipine significantly increased heart rate probably due to an activation of sympathetic nerves. Amlodipine (45 mg kg^{-1}) dose-dependently increased PRA up to six fold and renin mRNA levels up to seven fold.

Mibefradil exerted a dual effect on basal renin secretion and renin gene expression *in vivo*. At 5 mg kg^{-1} day^{-1}, mibefradil clearly reduced PRA and also moderately but significantly lowered renin mRNA levels. Increasing the daily dose of mibefradil to 45 mg kg^{-1} caused a moderate increase in PRA and renin mRNA levels. However, at each dose the stimulant effect of mibefradil of renin was far smaller than that of amlodipine. In isolated renal juxtaglomerular cells neither amlodipine nor mibefradil stimulated renin secretion, suggesting that the effects of both drugs *in vivo* on the renin system were indirect. In the concentration range of 0.1–10 μM amlodipine and mibefradil would be expected to block L-type and T-type calcium channels respectively (Mishira & Hermsmeyer, 1994; Koidl *et al*., 1997). It was notable that lowering extracellular calcium concentration stimulated renin secretion from cultured juxtaglomerular cells, which supports the conclusion that neither L-type nor T-type calcium channels are effectively involved in the control of renin secretion at the cellular level.

The inhibitory effect of low mibefradil doses on the renin system was exerted in situations where the renal renin system was stimulated. Such a stimulation was induced by unilateral renal hypoperfusion due to placement of a renal artery clip. Two days after placement of the renal artery clips PRA was increased about 4 fold and renin mRNA was increased about 5 fold in the clipped kidney, whilst renin mRNA was markedly suppressed in the contralateral, normal kidney. Pretreatment of animals with amlodipine (15 mg kg^{-1} day^{-1}) before renal artery clipping additionally elevated the rise of PRA and of renin mRNA. Mibefradil (5 mg kg^{-1} and 15 mg kg^{-1}), on the other hand, led to a clear attenuation of the rise of PRA and of renin mRNA in response to renal artery clipping.

By which indirect pathways amlodipine may stimulate and mibefradil may inhibit the renin system *in vivo* remains unclear. Apart from blood pressure, important determinants for the activity of the renin system *in vivo* comprise the salt load of the body (Holmer *et al*., 1993; Schricker *et al*., 1996b), sympathetic outflow (Holmer *et al*., 1994), a yet unidentified macula densa signal (Modena *et al*., 1993) as well as local factors such as intrarenal formation of angiotensin II, prostaglandins or nitric oxide (Johns *et al*., 1990; Schricker *et al*., 1995a,b).

Although our study cannot clearly distinguish between these various possibilities an obvious candidate could be sympathetic nerve activity. The changes of heart rate observed could be interpreted as a stimulant and an inhibition of sympathetic nerve activity by amlodipine and mibefradil, respectively. Thus it will be of interest to characterize the role of the sympathetic nervous system in the effects of calcium channel blockers on the renin system *in vivo*.

The expert technical assistance provided by K.H. Götz and M. Hamann is gratefully acknowledged.

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(Received April 30, 1997
Revised February 23, 1998
Accepted February 26, 1998)