Interactions between calcium antagonists and the sympathetic nervous system in various pharmacological models
van der Lee, R.

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Chapter 2

COMPARATIVE EFFECTS OF MIBEFRADIL AND OTHER CALCIUM ANTAGONISTS ON RESISTANCE ARTERIES OF DIFFERENT END ORGANS
1. Introduction

In vascular smooth muscle cells (VSMC) at least two types of calcium channels have been found to occur: the L-type calcium channels (LCC) and the T-type calcium channels (TCC) [1]. The LCC can be characterized by their relatively long lasting time of opening (300-600ms), activation range from -40 mV to positive voltage and large conductance (25 pS). On the other hand TCC display a much shorter time of opening (20-60ms), a low activation range (from -70 mV to -30 mV) and they show rapid inactivation and low conductance (8 pS) [19]. The LCC have been studied extensively and their structure, function and specific binding sites are well known. The LCC are known to be the target of the calcium antagonistic drugs so far developed [24].

For a long time TCC could only be characterized functionally by means of patch-clamp studies [2]. Only very recently the first TCC has been cloned from neuronal tissue of the rat [20]. TCC have been found in a variety of excitable cells including VSMC, cardiac myocytes and sino-atrial node cells [7]. Its function, however, still remains subject to debate.

Mibefradil is a newly introduced calcium antagonist (CA) with a verapamil-like chemical structure [5]. It has been proven effective in the treatment of hypertension and angina pectoris, and it also possesses anti-arrhythmic activity. Although mibefradil is a vasodilator which also causes bradycardia, it appears to be virtually devoid of negative inotropic activity in the clinically used dosage [3,22].

Mishra and Hermsmeyer (1994) reported for mibefradil an approximately 56-fold stronger inhibition of T-type vs. L-type Ca\(^{2+}\)-channels (TCC vs. LCC) in an electrophysiologic study with vascular smooth muscle cells (VSMC) [14]. However, the functional role of TCC blockade in the various cardiovascular effects of mibefradil remains unclear. Högestätt and Andersson (1983) proposed a new type of calcium channel which could be responsible for the phasic response of the potassium-induced contraction in rat isolated small arteries [11]. The functional profile of this calcium channel closely resembles the TCC as has been described by Nilius et al. [18].

The aim of the present investigation was to obtain information on the question whether the the vasodilator effect of mibefradil is associated with blockade of TCC, apart from the drug’s effect on LCC. Therefore we studied the influence of mibefradil on both the tonic and phasic components of potassium-induced contractions in different vascular beds of the male Wistar rat, and compared it with selective LCC-blockers. Accordingly, contractions induced by potassium depolarization were evoked in rat isolated mesenteric, renal, coronary and basilar arteries. The relaxant effects of various calcium antagonists (including mibefradil) on this vasoconstrictor response were then quantified and compared.
Since hypertension may influence the distribution of the different types of calcium channels, we included the mesenteric vessels of SHR and their respective normotensive control WKY in the present study.

2. Materials and methods

Male Wistar rats weighing 300-350g (Iffa Credo, Les Oncins, France) were anesthetized using a combination of ketamine (40mg i.p.) and xylazine (4mg i.p.) and subsequently they were given heparin (2500 IE i.p.). The animals were ventilated with room air via an intra-tracheal cannula. Part of the intestine with the adjacent mesenterium, the right kidney, the heart and the brain were excised and placed immediately in ice-cold Tyrode’s solution of the following composition (mM): NaCl 136, KCl 2.5, MgCl₂ 0.5, CaCl₂ 1.8, NaH₂PO₄ 0.42, NaHCO₃ 11.9, glucose 5.5. The fatty tissues surrounding the mesenteric artery were removed. The right kidney was cut in half and an arcuate artery was dissected free from the surrounding tissue over a length of about 2mm. After an incision in the right cardiac ventricle the coronary septal artery was dissected over a length of 2mm and separated from the surrounding myocardial tissue. The basilar artery was separated from the brain stem. Before dissection a 40μm diameter stainless steel wire was inserted into the arterial lumen of each vessel to be studied. Subsequently, the vessels were dissected and transferred to the chamber of a myograph according to Mulvany and Halpern [16]. The vessel was attached to a micrometer screw and, after insertion of a second wire, to an isometric force transducer (Kistler Morse, DSG 6, Redmond, WA, USA). The preparations were equilibrated for 15 min. in Tyrode’s solution at 37°C and the medium was oxygenated with carbogen (95% O₂ + 5% CO₂), at a pH of 7.4. Subsequently the vessels were subjected to a normalization procedure according to Mulvany and Halpern (1976). The individual diameter was adjusted to a value that equals 90% of the circumference that a vessel would have at a transmural pressure of 100 mmHg (13.3kPa) [17]. Mechanical responses were expressed as active tension, ΔT, that is the developed active force divided by twice the vessel length. The responsiveness and the viability of the vessels were tested as follows: at the beginning of each experiment, the preparations were contracted three times with high-potassium Tyrode’s solution (NaCl was replaced by KCl on an equimolar basis) and once with 3 μM 5-hydroxytryptamine (5-HT) at intervals of 15 min. After these procedures the vessels were incubated with the CA to be studied for 30 min. and then subjected (in the presence of the CA) to a potassium-induced contraction. Accordingly we were able to measure the inhibition of the biphasic contraction by the CA studied. CA-concentration-response curves (CRC) were constructed for the phasic and tonic responses, respectively.
WKY and SHR (weighing 300-350g) were anesthetized and ventilated according to the protocol described above. After insertion of a cannula (PE-50, Clay Adams) into the right carotid artery heparin (250 IU) was administered to keep the cannula open. Carotid blood pressure and heart rate were measured intra-arterially by a Statham P23 Db pressure transducer and recorded via a Maclab data acquisition system (ADI, Australia). Then the rats were subjected to the same protocol as the Wistar rats, but only the small intestine was removed in order to study the effect of nifedipine and mibefradil on small mesenteric arteries.

2.1. Statistical evaluation
Unless stated otherwise the values in the text are given as means ± standard error of the mean (SEM). Statistical significance was established by one-way ANOVA, followed by Newman-Keuls’ test for multiple comparisons. The level of significance was set at p<0.05.

2.2. Calculations
Using a computer program (GraphPad Prism, GraphPad, San Diego, USA) all curves were fitted to log concentration-effect data for 4-10 individual experiments. The underlying equation is \( E = E_{\text{max}} \cdot A^p \cdot (A^p + IC_{50}^p)^{-1} \). In this equation \( E \) is the response obtained at a given concentration \( A \), \( E_{\text{max}} \) is the maximally attainable response, \( IC_{50} \) the concentration for the half maximal effect, and the exponent \( p \) describes the slope of the relationship (Hill-coefficient). Curves were fitted to averaged concentration-effect data.

2.3. Drugs used
The following drugs were used: ketamine (Nimatek®, Eurovet, Bladel, The Netherlands); xylazine (Rompun®, Bayer bv, Leverkusen, Germany); heparine sodium (Heparine Leo, Leo Pharmaceutical products bv, Weesp, The Netherlands); 5-hydroxytryptamine, nifedipine HCl, verapamil HCl, and diltiazem (Sigma Chemical Co., St. Louis, MO, USA) and mibefradil (Hoffman-LaRoche, Basel, Switzerland).

5-Hydroxytryptamine, verapamil, diltiazem and mibefradil were dissolved and further diluted in distilled water; nifedipine was first dissolved in 67% dimethyl sulfoxide (DMSO) and then further diluted with distilled water. Experiments with nifedipine were performed under the exclusion of light.
3. Results

3.1. Wistar rats

Both the diameter and the active force induced by potassium depolarization of the vessels were determined and compared (table 1). The normalized internal diameters of the four types of vessels were virtually the same, but significant differences were observed with respect to the development of the active force, induced by potassium depolarization. For the active force the following rank order was found: basilar>mesenteric>renal, coronary.

Potassium-induced depolarization resulted in a rapidly developing and transient first phase (phasic response) followed by a stable second phase (tonic response). The profiles of the obtained responses were typical for each of the types of vessels studied (figure 1). All four CA reduced potassium-induced responses in all types of vessels investigated. The phasic responses were less potently inhibited than the tonic responses as shown in figure 2, where an example of the CRC's obtained for one of the CA (nifedipine) is depicted. At 0.3μM of each CA the tonic phase was completely inhibited by all CA. However, at this concentration the phasic response could not be fully inhibited as shown in figure 3. Nifedipine is clearly the most efficient inhibitor of the phasic response with 66-77% reduction. The effect of mibefradil was less effective (12-50% reduction), but comparable to that of verapamil (20-38%) and diltiazem (10-28%). Nifedipine also proved to be the most potent inhibitor of the tonic response, compared to the other CA tested. This is reflected by the order of potency: nifedipine > mibefradil > verapamil > diltiazem. A comparison can be made between the IC_{50}-values of the CA in the different vascular beds. Nifedipine and verapamil appeared to display a significant preference for the mesenteric and renal vascular beds; mibefradil proved somewhat more potent for the mesenteric vascular bed whereas diltiazem is most active in renal and coronary vessels (table 2).

### Table I. Basal characteristics of the different types of isolated vessel preparations from Wistar rats.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>N</th>
<th>Diameter (μm)</th>
<th>Force (mN/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mesenteric</td>
<td>28</td>
<td>283.4 ± 6.7</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>renal</td>
<td>24</td>
<td>299.8 ± 8.9</td>
<td>1.5 ± 0.1*</td>
</tr>
<tr>
<td>coronary</td>
<td>24</td>
<td>287.7 ± 6.8</td>
<td>1.7 ± 0.1*</td>
</tr>
<tr>
<td>basilar</td>
<td>28</td>
<td>303.3 ± 8.4</td>
<td>3.0 ± 0.1</td>
</tr>
</tbody>
</table>

Means ± SEM for n preparations. The active force was induced by means of potassium depolarization. *significantly different from the responses in both mesenteric and basilar preparations (p<0.05).
Figure 1. Characteristic responses for each type of vessel caused by potassium-induced depolarization. Note the rapid first phase (phasic response, P) and the longer lasting second phase (tonic response, T).

Table II. Relaxant effects of various CA on the tonic response of potassium-induced contractions in isolated vessel preparations. The IC\textsubscript{50} values calculated from the CRC's are given in nM ± SEM for n preparations.

<table>
<thead>
<tr>
<th></th>
<th>mesenteric (n=7)</th>
<th>Renal (n=6)</th>
<th>coronary (n=6)</th>
<th>basilar (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nifedipine</td>
<td>1.1 ± 0.1*</td>
<td>1.2 ± 0.1*</td>
<td>4.0 ± 0.4</td>
<td>5.4 ± 0.8</td>
</tr>
<tr>
<td>Mibebradil</td>
<td>19.6 ± 6.0\dagger</td>
<td>77.6 ± 21.5</td>
<td>63.0 ± 9.2</td>
<td>60.6 ± 6.8</td>
</tr>
<tr>
<td>Verapamil</td>
<td>54.5 ± 8.2\dagger</td>
<td>37.7 ± 4.3\dagger</td>
<td>135.7 ± 22.1</td>
<td>178.9 ± 21.1</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>322.0 ± 55.1</td>
<td>263.8 ± 49.2\dagger</td>
<td>202.0 ± 39.6\dagger</td>
<td>470.5 ± 65.6</td>
</tr>
</tbody>
</table>

* significantly different from coronary and basilar preparations (p<0.001); † significantly different from renal preparations (p<0.05); ‡ significantly different from coronary and basilar preparations (p<0.01); ‼ significantly different from basilar preparations (p<0.05)
Table III. Hemodynamic parameters and vessel characteristics of the mesenteric vessels of WKY and SHR.

<table>
<thead>
<tr>
<th>parameter</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>305 ± 3</td>
<td>303 ± 5</td>
</tr>
<tr>
<td>Vessel diameter (µm)</td>
<td>269 ± 10</td>
<td>217 ± 13*</td>
</tr>
<tr>
<td>Active Force (mN/mm)</td>
<td>3.0 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>98 ± 5</td>
<td>167 ± 6*</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>281 ± 14</td>
<td>289 ± 14</td>
</tr>
</tbody>
</table>

*Means are significantly different from WKY (p<0.05)

Figure 2. Typical concentration-response curve for the relaxant effect of a calcium antagonist (nifedipine) on the potassium-induced contraction in isolated mesenteric vessels; note the differential effect of the CA on the phasic and tonic responses, respectively (squares: phasic response; triangles: tonic response).

3.2. SHR and WKY

The hemodynamic parameters and vessel characteristics of the WKY and SHR are presented in Table 3. Beside the difference in MAP, a significant difference in the normalized internal diameters of the vessels between WKY, SHR and Wistar rats was found. The active force was comparable between the three groups of rats. The effects of nifedipine and mibebradil on the tonic (IC₅₀-values) and phasic responses are presented in Table 4. For both nifedipine and mibebradil, IC₅₀-values in WKY and SHR were significantly higher than in Wistar rats (p<0.001) and the difference between WKY and
Calcium antagonists and resistance arteries

SHR was significantly different (p<0.05). Like in Wistar rats the phasic response of the KCl-induced depolarization could not be inhibited completely.

Table IV. Inhibitory effects of nifedipine and mibefradil in SHR and WKY on the tonic (IC50-values) and phasic responses (IPR), respectively (n=6 for each experiment).

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>nifedipine</th>
<th>SHR</th>
<th>WKY</th>
<th>mibefradil</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (nM)</td>
<td>4.1 ± 0.6</td>
<td>5.5 ± 0.6*</td>
<td>34 ± 4</td>
<td>50 ± 4*</td>
<td></td>
</tr>
<tr>
<td>IPR (%)</td>
<td>67 ± 6</td>
<td>52 ± 6*</td>
<td>30 ± 4</td>
<td>21 ± 4*</td>
<td></td>
</tr>
</tbody>
</table>

* means significantly different from WKY (p<0.05)

Figure 3. Influence of various CA on the phasic response of potassium-induced contractions in mesenteric (open), renal (shaded), coronary (hatched) and basilar (filled) isolated vessel preparations. All CA were administered in a concentration of 0.3μM. Depicted is the relative inhibition of the potassium-induced contraction after application of the CA (n = at least 6 for each experiment).

4. Discussion

Small arteries (range 100-500μm) are known to be important in the regulation of peripheral vascular resistance [6]. In the present investigation the vessels studied had normalized internal diameters slightly under 300μm (which is about twice the vessel diameter in resting state) thus classifying them as small arteries [15]. As such they are suitable and relevant to study the vasodilator action of CA in resistance vessels. From a
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In 1983 Högestätt and Andersson investigated the biphasic contraction caused by potassium-induced depolarization in rat small cerebral arteries. They concluded that the tonic response was clearly LCC mediated, whereas for the phasic response a different type of calcium channel could be responsible; the profile of their proposed calcium channel closely resembles what is now known as the TCC. The differentiation between LCC- and TCC-mediated effects would offer the possibility to identify and possibly quantify a TCC-blocking component in the profile of action of the CA investigated. The difference between the activation ranges of the LCC and the TCC is indeed substantial: -40 mV to positive vs. -70 mV to -30 mV. This indicates that the TCC can only be involved in the phasic response. In our experiments nifedipine was by far the most potent inhibitor of the phasic response. Mibefradil proved as effective as verapamil and diltiazem (rather selective blockers of the LCC) in blocking the phasic response. Since mibefradil is not exclusively a TCC-antagonist, we cannot absolutely exclude TCC in the phasic response. It should be realized therefore, that mibefradil displays substantial LCC-blocking activity besides its effect on TCC. However, the fact that nifedipine (a selective LCC-antagonist) is by far the most potent inhibitor of the phasic response strongly suggests that the vasodilator effects of the CA (including mibefradil) were mediated by LCC rather than by TCC. Since hypertension leads to several major changes in the vasculature [9,10,12,13], the distribution of the different types of calcium channels in this situation might be different. Since most vascular changes in hypertension occur in mesenteric vessels, which are also determinant with respect to pressure and peripheral resistance, we chose this vessel as model in our experimental setup. However, in our experiments with WKY and SHR we could not demonstrate a preference of mibefradil for the phasic component of the KCl-induced contraction. From these results we conclude that the TCC does not play a role in the excitation-contraction coupling in vascular smooth muscle under the conditions of the present investigation.

The tonic phase has been shown to depend predominantly on calcium influx via LCC. Accordingly, IC$_{50}$-values of the four CA, with respect to their influence on the tonic phase, yield information concerning the vasodilator potency of these agents mediated by LCC blockade.

Nifedipine was the most potent vasodilator with respect to the tonic response compared to the other CA tested. Again, the vasodilator capacity of mibefradil was comparable with those of verapamil and diltiazem, as observed in the Wistar rats. Accordingly, mibefradil appears to possess sufficient LCC blocking activity to be a maximally effective vasodilator in the various vascular beds.

The differences in IC$_{50}$-values for both nifedipine and mibefradil between WKY and SHR might suggest that hypertension leads to a decrease in sensitivity of the LCC to

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technical point of view the preparations used proved reliable and the effects were reproducible.
these CA. However, it should be realized that normalizing the vessel preparations of WKY and SHR to the same intraluminal pressure may yield an experimental error, since the vessels of SHR in vivo are exposed to a higher pressure than the vessels of WKY. There is also a consistent difference between the IC_{50}-values of SHR and WKY versus Wistar rats: responses are significantly stronger in Wistar rats. Since our experimental conditions were kept exactly the same throughout the study, the observed differences might be attributable to genetic differences between the three types of rats.

The introduction of the TCC/LCC-blocker mibefradil into the therapy of hypertension and angina pectoris has stimulated the research on TCC in the cardiovascular system. The TCC has been identified in several kinds of tissues, including VSMC [23]. However, the role of TCC and their blockade in VSMC is not clear [8]. In the cardiovascular system the TCC are likely to be involved in at least two processes. They have been demonstrated to occur in the conductive system in the heart they may play a role in ventricular arrhythmias [3], secondly they have been implicated in neointima formation following vascular injury [21]. If these presumptions hold true mibefradil would combine a moderate vasodilator activity with protective effects against both cardiac arrhythmias and neointima formation, whereas it is virtually devoid of negative inotropic activity. The clinical value of this profile would be potentially attractive, although appropriate studies to demonstrate the advantage of mibefradil over other CA remain to be performed.

There are reports that mibefradil might be somewhat selective for coronary arteries, but in the present study we did not observe this selectivity. Only diltiazem showed a certain preference for the coronary vessels, which is in agreement with literature data [4].

In conclusion, the vasodilator action of mibefradil as investigated in this study can be fully explained by its inhibitory effect on LCC in normotensive rats. This statement can be extended to both normotensive and hypertensive rats with regard to mesenteric resistance arteries. No involvement of TCC in the phasic component of the potassium-induced contraction could be detected.

References


3. Billman GE, Hamlin RL. The effects of mibefradil, a novel calcium channel antagonist on ventricular arrhythmias induced by myocardial ischemia and programmed electrical stimulation. *J Pharmacol Exp Ther* 1996; 277: 1517-1526


