Interactions between calcium antagonists and the sympathetic nervous system in various pharmacological models
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Chapter 5

INHIBITORY EFFECT OF MIBEFRADIL ON CONTRACTIONS INDUCED BY SYMPATHETIC NEUROTRANSMITTER RELEASE IN THE RAT TAIL ARTERY

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1. Introduction

Mibefradil is a calcium antagonist which is capable of blocking at least three types of calcium channels in the micromolar concentration range: the L-, T- and N-type calcium channels [1]. Because of this property mibefradil continues to be of scientific interest, despite the withdrawal of the drug in 1998 from the market because of several interactions with drugs also metabolized through the cytochrome P450 enzymatic system. The antihypertensive and anti-ischaemic activities of mibefradil are probably based predominantly upon its capability to block L-type calcium channels [2]. The role and relevance of the T-type calcium channel and its blockade by mibefradil continues to be subject of debate.

The N-type calcium channel is involved in the release of neurotransmitters from sympathetic nerves [3], and for this reason the blockade of this channel with calcium antagonists may be expected to suppress prejunctional noradrenaline release. Accordingly, Göthert and Molderings showed in 1997 that mibefradil could inhibit ω-conotoxin GVIA-sensitive sympathetic noradrenaline-release in human right atrial appendages. Furthermore, the negative chronotropic activity of mibefradil was attributed by these authors at least partially to the inhibition of noradrenaline-release in the right atrium [4]. These findings in cardiac atrial tissue prompted us to study a comparable issue in vascular smooth muscle preparations.

In blood vessels, the density of sympathetic innervation varies according to the type, location and function of the vessel. The physiological role of sympathetic innervation of blood vessels is twofold: first it participates in the regulation of basal vascular tone and furthermore it provides a rapid response system to varying hemodynamic needs. Several neurotransmitters, such as noradrenaline, ATP and neuropeptide Y are known to contribute to the constrictor responses elicited by perivascular sympathetic nerve stimulation [5-9]. In the rat, one of the most densely sympathetically innervated blood vessels is the tail artery. Electrical field stimulation (EFS) is known to provoke a discharge of the neurotransmitters stored in the vesicles of the sympathetic nerve terminals [10,11]. It therefore seemed of interest to use this technique in order to study the influence of calcium antagonists on the neurogenic constrictor responses. Accordingly, we compared the effects of mibefradil and verapamil (an L-type calcium channel blocker with no known affinity for the N-type calcium channel) on responses of the rat tail artery to endogenous neurotransmitter release provoked by EFS. Moreover we compared the actions of both calcium antagonists on responses to the exogenously applied neurotransmitters noradrenaline and ATP.
2. Methods

Male Wistar rats weighing 267 ± 8g (Broekman, The Netherlands) were stunned by a blow on the head and then decapitated. The tail was cut off at the base and 3cm of the tail artery was exposed 3cm down from the base. Four arterial segments with a length of 2mm each were prepared and a stainless steel wire with 40μm diameter was inserted into each vessel; the segments were then transferred to the organ bath of an isometric wire myograph. The organ bath contained 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. Ascorbic acid (100mg/L) was added to prevent oxidation of noradrenaline. Propranolol (1μM), L⁵⁻N-Nitro Arginine (L-NNA) (1μM) and indomethacin (1μM) were added in order to exclude the β-adrenergic effects of noradrenaline and the effects of endothelium-derived NO and prostaglandins. The preparations were 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 equilibrated with a mixture of 95% O₂ and 5% CO₂, at a pH of 7.4. Subsequently the vessels were subjected to a normalization procedure according to Mulvaney and Halpern (1976) [12]. The individual circumference was adjusted to 90% of the value the particular vessel would have had at a transmural pressure of 100mmHg (13.3kPa) . Mechanical responses were expressed as developed active force (ΔF).

Electrical Field Stimulation (EFS) was applied using thin platinum wire-electrodes positioned on either end of the vessel. Contractions were generated using a current of alternating 85/-85 mA with a pulse width of 0.2ms for 30sec per frequency step. The frequency steps used in EFS were 0.25, 0.5, 1, 2 and 4Hz, respectively. Henceforth, the term EFS implies that the full range of frequency steps was applied in a cumulative way.

2.1. Experimental protocol, EFS

After the 15min equilibration period a priming procedure consisting of twice applied EFS with an interval of 15min initiated the protocol. Subsequently a baseline EFS was carried out which served as reference to the values obtained in the presence of the calcium antagonists. After the baseline EFS the medium was changed with Tyrode’s solution containing one particular concentration of the calcium antagonists to be tested. After an incubation period of 15min three times EFS with intervals of 15min were performed in order to evaluate the effect of the calcium antagonists. The third EFS-
induced contractions were used for further analysis. Control experiments with tetrodotoxin (1μM), guanethidine (3μM) suramin (500μM) and prazosin (3μM) were performed in order to check whether the contractions were neuronally mediated and possibly caused by a co-transmission of noradrenaline and ATP.

2.2. Experimental protocol, noradrenaline and ATP

The priming procedure consisted of the twofold, subsequent application of a concentration of either noradrenaline (1μM) or ATP (0.3mM), respectively, with intervals of 15min each. Subsequently, a concentration-response curve (CRC) was constructed for noradrenaline (concentration range 0.03-3μM) or a contraction was induced with 0.3mM of ATP (5min) which served as a reference value. After incubation for 15min with one particular concentration of the calcium antagonist to be tested, three consecutive CRC's for noradrenaline or three times a contraction caused by 0.3mM of ATP (5min) were generated in the presence of the calcium antagonist. The third CRC or contraction was used as the basis for further analysis.

2.3. Statistical evaluation

Unless stated otherwise the values in the text are given as means ± standard error of the mean (SEM). Statistical significance was evaluated by one-way ANOVA, followed by Newman-Keuls' test for multiple comparisons, or Student's t-test when appropriate. The level of significance was set at p<0.05. Using a computer program (GraphPad Prism, GraphPad, San Diego, USA) all curves were fitted to log concentration-effect data for 4-6 individual experiments. The underlying equation is $E = E_{max} \cdot A^p \cdot (A^p + IC_{50}^p)^{-1}$. In this equation E is the response obtained at a given concentration A. $E_{max}$ 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). For each individual experiment a CRC was fitted and mean $E_{max}$ and $IC_{50} \pm$ SEM were calculated, along with the corresponding p-values.

2.4. Drugs used

The following drugs were used: propranolol HCl (Imperial Chemical Industries Ltd., Macclesfield, Cheshire, UK), L-NNA HCl, tetrodotoxin, verapamil HCl (Sigma Chemical Co., St. Louis, MO, USA), indomethacin (Merck Sharp and Dohme, Haarlem, The Netherlands), noradrenaline bitartrate (Hoechst, Amsterdam, The Netherlands), ATP (Boehringer, Mannheim, Germany), guanethidine, prazosin HCl (Pfizer, Brussels.
Belgium), suramin hexasodium (Research Biochemicals International, Natick, MA, USA). Mibefradil was kindly donated by Hoffman-LaRoche, Basel, Switzerland.

3. Results

3.1. Basal parameters
The normalized diameter of the vessels averaged 620 ± 9 µm. EFS induced contractions with a maximal force of 15 ± 1 mN (n=6) at a stimulation frequency of 4 Hz. In control experiments stable responses could be obtained for at least six consecutive EFS-induced contractions (n=6) or at least six consecutive CRC of either noradrenaline or six contractions with 0.3 mM of ATP (n=6), respectively.

Table 1. Electrical field stimulation-induced contractions in isolated preparations of the rat tail artery: force generation and the inhibitory effects of prazosin and suramin. Data are presented as percentages ± SEM. (n=6)

<table>
<thead>
<tr>
<th>EFS freq. (Hz)</th>
<th>Force (mN)</th>
<th>prazosin 3µM (% inhibition)</th>
<th>suramin 0.5mM (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>5 ± 0.5</td>
<td>97 ± 0.3</td>
<td>0</td>
</tr>
<tr>
<td>0.50</td>
<td>7 ± 0.7</td>
<td>97 ± 0.3</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>1.00</td>
<td>10 ± 0.8</td>
<td>94 ± 0.6</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>2.00</td>
<td>14 ± 0.8</td>
<td>85 ± 1.0</td>
<td>13 ± 0.9</td>
</tr>
<tr>
<td>4.00</td>
<td>15 ± 0.9</td>
<td>67 ± 3.2</td>
<td>31 ± 3.0</td>
</tr>
</tbody>
</table>

3.2. Electrical Field Stimulation and Calcium Antagonists
In control experiments, tetrodotoxin (1µM) and guanethidine (3µM) abolished the contractions evoked by EFS. Prazosin (3 µM) prevented responses to the lower frequencies completely, whereas the EFS-induced contractions to 2 and 4 Hz were inhibited by 85 ± 1% and 67 ± 3%, respectively. The remaining component could be inhibited by 500 µM suramin, a P2-receptor antagonist. Table 1 summarizes the results obtained in these control experiments.

Mibefradil was able to block the EFS-induced contractions by nearly 100% at a concentration of 10 µM at all stimulation frequencies used. In contrast, verapamil could
not completely block EFS-induced contractions at any of the frequencies used. Fig. 1 demonstrates the difference between mibefradil and verapamil (both at a concentration of 10 μM) with respect to the inhibition of the EFS-induced contractions. Fig. 2 depicts the inhibitory effect of both calcium antagonists on contractions induced at a stimulation frequency of 1 Hz. In Table 2 the results of the experiments with mibefradil and verapamil are listed.

**Figure 1.** Contraction induced by electrical field stimulation (EFS) in isolated rat tail artery preparations. Inhibitory effects of mibefradil and verapamil (10μM) on EFS-induced contractions are shown. Data are given as means (mN) ± SEM (n=5-6).

**Figure 2.** Concentration-dependent inhibition of contractions induced by electrical field stimulation (EFS) at a frequency of 1Hz is shown for both mibefradil and verapamil. Data are given as means (%) ± SEM (n=5-6). Note that mibefradil achieves nearly 100% inhibition, whereas verapamil does not (p<0.05).
3.3. Exogenous Noradrenaline and Calcium Antagonists
The concentration range of noradrenaline used (0.03-3μM) generated a reliable CRC. The maximum force generated by noradrenaline was 14 ± 1mN (n=6) at a concentration of 3μM. Neither of the two calcium antagonists was able to inhibit the noradrenaline-induced contractions completely; the maximum inhibition of the response to 1μM noradrenaline with the maximum concentration used of either calcium antagonist was 70 ± 6% and 75 ± 5% for mibebradil and verapamil, respectively. The logIC₅₀-values of mibebradil and verapamil for inhibiting the effects of 1μM noradrenaline were -6.7 ± 0.1 and -6.5 ± 0.1, respectively. These differences were not statistically different (p>0.05), as depicted in Fig. 3. Table 3 contains the results of the experiments with noradrenaline and both calcium antagonists at a concentration of 10μM.

Table 2. The inhibitory action of 10μM of mibebradil and verapamil on contractions induced by electrical field stimulation in isolated rat tail artery preparations is given as percentages ± SEM. Furthermore the logIC₅₀-values ± SEM of both calcium antagonists (applied at concentration ranges of 0.3 to 10 μM) are given for each stimulation frequency (n=5-6). EFS = electrical field stimulation, MIB = mibebradil, VER = verapamil.

<table>
<thead>
<tr>
<th>EFS freq. (Hz)</th>
<th>MIB 10μM (% inhibition)</th>
<th>VER 10μM (% inhibition)</th>
<th>MIB logIC₅₀</th>
<th>VER logIC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>93 ± 3*</td>
<td>82 ± 4</td>
<td>-5.4 ± 0.1*</td>
<td>-6.8 ± 0.9</td>
</tr>
<tr>
<td>0.50</td>
<td>96 ± 2*</td>
<td>79 ± 5</td>
<td>-5.6 ± 0.1*</td>
<td>-6.9 ± 0.8</td>
</tr>
<tr>
<td>1.00</td>
<td>97 ± 1*</td>
<td>73 ± 4</td>
<td>-5.6 ± 0.1*</td>
<td>-6.6 ± 0.2</td>
</tr>
<tr>
<td>2.00</td>
<td>98 ± 1*</td>
<td>64 ± 0.4</td>
<td>-5.6 ± 0.1*</td>
<td>-6.5 ± 0.1</td>
</tr>
<tr>
<td>4.00</td>
<td>98 ± 1*</td>
<td>55 ± 2</td>
<td>-5.7 ± 0.1*</td>
<td>-6.7 ± 0.4</td>
</tr>
</tbody>
</table>

* means p<0.05 vs. VER

3.4. ATP and Calcium Antagonists
The maximum force of contraction generated by 0.3mM ATP in control experiments was 8 ± 0.5 mN (n=6). Both calcium antagonists at their maximum concentration (10μM) were capable of blocking more than 90% of the contraction generated by 0.3 mM ATP: mibebradil by 92 ± 1% and verapamil by 97 ± 1% (n.s.) The logIC₅₀-values were -6.5 ± 0.1 and -7.0 ± 0.1 for mibebradil and verapamil, respectively (p<0.05).
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Figure 3. Inhibition of contractions induced by exogenously applied noradrenaline (1μM) is shown for the full concentration range used for both mibefradil and verapamil. Data are given as means (%) ± SEM (n=6). Note that neither of the two CA achieves maximal inhibition.

Table 3. The inhibitory effect of 10μM mibefradil and verapamil on contractions generated by exogenously applied noradrenaline in rat isolated tail artery preparations is given as percentages ± SEM and the force developed in response to the various noradrenaline concentrations is given in mN± SEM. Furthermore, the log IC₅₀-values (± SEM) of both calcium antagonists (applied at concentration ranges of 0.3 to 10 μM) are given for the noradrenaline concentrations of 0.3, 1 and 3 μM (n=6 in all series of experiments). NA = noradrenaline, MIB = mibefradil, VER = verapamil, n.a. = not available (due to low yield of force).

<table>
<thead>
<tr>
<th>NA conc. (logM)</th>
<th>Force (mN)</th>
<th>MIB 10μM (% inhibition)</th>
<th>VER 10μM (% inhibition)</th>
<th>MIB logIC₅₀</th>
<th>VER logIC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>-7.5</td>
<td>0.1 ± 0.02</td>
<td>40 ± 24</td>
<td>40 ± 24</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>-7.0</td>
<td>1.0 ± 0.1</td>
<td>51 ± 9</td>
<td>60 ± 17</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>-6.5</td>
<td>4.9 ± 0.5</td>
<td>67 ± 6</td>
<td>83 ± 6</td>
<td>-6.5 ± 0.2</td>
<td>-6.4 ± 0.1</td>
</tr>
<tr>
<td>-6.0</td>
<td>10.8 ± 0.6</td>
<td>70 ± 6</td>
<td>75 ± 5</td>
<td>-6.7 ± 0.1</td>
<td>-6.5 ± 0.1</td>
</tr>
<tr>
<td>-5.5</td>
<td>14.1 ± 0.7</td>
<td>59 ± 5</td>
<td>60 ± 3</td>
<td>-6.1 ± 0.1</td>
<td>-6.1 ± 0.1</td>
</tr>
</tbody>
</table>

4. Discussion

EFS as a method to mimick sympathetic nerve activation has been used for over 30 years [13]; despite this long time no standard procedure has been established. Differences include voltage- instead of ampere-fixed setups, wide ranges in the
frequencies used and even the setup of the electrodes: on either side or on either end of the vessel. The method we have used has been validated by the group of DeMey [14].

In general, EFS leads to membrane depolarization of the sympathetic nerve terminals, thereby opening the N-type calcium channels. The rise in intracellular calcium triggers the release of neurotransmitters stored in large dense-cored vesicles (LDV) and small dense-cored vesicles (SDV) [5]. Bao et al. (1990) showed that EFS applied to the rat tail artery leads to the release of noradrenaline and ATP, resulting in the stimulation of α₁- and P₂x-receptors with subsequent smooth muscle contraction [15].

As shown in our control experiments, the contractions elicited by EFS were neuronally mediated and fully caused by release of noradrenaline and ATP, and these results are in accordance with literature data [15-17]. In our experiments with prazosin it was demonstrated that contractions provoked by EFS with frequencies up to 1 Hz were mediated by noradrenaline by at least 94%. Since it was the aim of our study to find out whether the calcium antagonists could impair neurogenic noradrenaline-release, we focussed our attention on the contractions elicited by stimulation at 1 Hz.

The most striking result of this study is the finding that mibefradil is able to block the EFS-induced contraction completely, as opposed to verapamil. Clearly mibefradil has additional properties allowing a complete inhibition of neuronally elicited contractions. Considering the evidence obtained in an electrophysiological study by Bezprozvanny and Tsien (1995) [1], it is very likely that at the concentrations used, mibefradil is able to block the N-type calcium channel, thereby preventing the release of noradrenaline and ATP.

P₂x-purinoceptors are cation channels capable of transporting calcium into the cytosol. Furthermore activation increases cation conductances, leading to excitatory junction potentials and finally to depolarization of the plasma membrane, resulting in the opening of L-type calcium channels [5]. In our study both calcium antagonists were able to inhibit contractions to exogenously applied ATP by more than 90%: apparently, vascular smooth muscle contraction mediated by activation of the P₂x-purinoceptor leads, indirectly, to depolarization resulting in the opening of L-type calcium channels. Accordingly, L-type calcium channel activation accounts for the major part of the contractile response to ATP in our experiments.

Stimulation of α₁-adrenoceptors can induce a rise in cytosolic calcium via at least three different pathways: 1. by activating IP₃ via phospholipase C, resulting in the release of calcium ions from intracellular stores; 2. by directly activating L-type calcium channels via a stimulatory G-protein followed by the influx of extracellular calcium into the cytosol [18]. The fact that mibefradil and verapamil cannot completely inhibit
contractions to exogenous applied noradrenaline strengthens the notion that the additional inhibitory effect of mibefradil on EFS-induced contractions might be attributed to prejunctional effects. The inhibition of EFS-induced contractions by verapamil becomes progressively less at higher stimulation frequencies. Probably this is caused by a shift in coupling of the α1-receptor to other G-proteins at increasing noradrenaline concentrations, thereby increasing PLC-activation relative to G-protein coupled L-type calcium channel activation. To summarize, in this study mibefradil and verapamil display comparable effects except on EFS-induced contractions, where mibefradil proved capable of complete inhibition as opposed to verapamil.

Bezprozvanny and Tsien (1995) showed in an electrophysiological study that mibefradil displays affinities for the α1A- (P/Q-type calcium channel), α1B- (N-type calcium channel), α1C- (L-type calcium channel) and α1E- (R-type calcium channel) subunits. The affinity for the α1B-subunit was the highest [1]. A high affinity of a calcium antagonist for the main subunit of a calcium channel does not automatically imply that this calcium antagonist is also capable of functionally blocking this channel, as the authors point out, since the additional subunits might alter affinities for calcium antagonists. However, there is accumulating evidence that the classical concept of the mode of action of the therapeutically used calcium antagonists, which are known to act predominantly as L-type calcium channel blockers, is not applicable anymore. Mibefradil has been shown capable of blocking T-, L- and N-type calcium channels at micromolar concentrations in functional and electrophysiological studies [4,19,20]. The additional capability of blocking the N-type calcium channel could explain part of its clinical profile: no reflex tachycardia in spite of its vasodilator action, and a distinct negative chronotropic activity (see Petrie et al., 1995) [21].

Verapamil is known to have a low affinity for the T-type calcium channel in addition to its high affinity for the L-type calcium channel. Verapamil is not known to have affinity for the N-type calcium channel. Amlodipine, an atypical 1,4-dihydropyridine (1,4-DHP) because it is ionized at physiological pH, was found to have an equal affinity for both the L-type and the N-type calcium channels in an electrophysiological study by Furukawa et al. (1997) [22]. Isradipine, another 1,4-DHP was reported to inhibit contractions generated by endothelin-1 in isolated vascular smooth muscle cells from the rabbit aorta via a nifedipine-insensitive steady-state, voltage dependent R-type calcium channel (Bkailly et al., 1995) [23].

Obviously, some of the calcium antagonists display a wider spectrum of activities than previously anticipated. With respect to the additional blockade of the N-type calcium channel, this mechanism might be considered as a beneficial property. The sympathetic
nervous system is known to be activated in several pathological conditions, such as hypertension and heart failure. Reducing the level of activation of the sympathetic nervous system along with lowering of the blood pressure is advantageous in the treatment of patients with these disorders.

References

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