Venous and arterial coronary artery bypass grafts in a pharmacological perspective
Rinia-Feenstra, M.

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CHAPTER 6

The effect of calcium antagonists in preventing saphenous vein spasm

M. Rinia-Feenstra, M. Pfaffendorf, B.A.J.M. de Mol, and P.A. van Zwieten

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Introduction

In aortocoronary bypass surgery (CABG), calcium antagonists (CA), which are potent vasodilators, are successfully applied to prevent and treat graft spasm. The common mode of action of CA is the inhibition of calcium entry through voltage-dependent calcium channels into cells. From the characterized voltage-dependent calcium channels, only long-lasting (L-type) and transient (T-type) channels have been identified in vascular smooth muscle cells (VSMC).\(^1\)\(^2\) N(euronal)-type calcium channels are involved in the activation of nerve endings present in the vessel wall, leading to the release of noradrenaline and subsequent vasoconstriction.\(^3\) Verapamil, a predominantly L-type calcium channel blocker, has been shown to prevent spasm of the saphenous vein (SV) when locally applied during its dissection.\(^4\) Furthermore, after coronary revascularization with arterial grafts, patients are treated systemically with nitroglycerine or CA (nifedipine, diltiazem) to prevent spasm of the grafts.\(^5\)\(^6\) Another possible beneficial effect associated with the use of CA is the inhibition of VSMC proliferation, since verapamil demonstrated this property in cells originating from graft or native SV, in vitro.\(^7\)

In recent years new CA have been developed to reduce negative side effects associated with the clinical use of these compounds (negative inotropic action, reflexstachycardia) and have been claimed to exert a higher vasoselectivity. One of these new CA, mibefradil (Ro 40-5967), blocks T-type Ca\(^{2+}\)-channel currents in VSMC at lower concentrations than those necessary for L-type current blockade.\(^8\) Furthermore, mibefradil exerted a stronger inhibitory effect than verapamil on sympathetic neurotransmission, presumably caused by N-type calcium channel blockade.\(^3\) Recently mibefradil has been demonstrated to posses an infarct size-limiting effect in the myocardium, which could be abolished by glibenclamide. These findings suggest that mibefradil might also activate K\(_{ATP}\)-channels in the myocardium.\(^9\) On VSMC of the injured rat carotid artery mibefradil demonstrated an antiproliferative effect. Rapidly proliferating cells express T-type calcium channels, mediating the increase of intracellular calcium induced by certain growth factors.\(^10\)

Since CA are applied to prevent arterial and venous graft spasm, and new compounds have been developed it seemed of interest to define the properties of CA necessary for an optimal prevention of graft spasm. Furthermore, little attention has been paid to the prevention of spasm of saphenous vein. Therefore, in the present study, verapamil, a classic CA, was compared to mibefradil, a CA with a particular pharmacological profile, in the human saphenous vein.
Materials and Methods

Human isolated saphenous vein preparations

Saphenous vein remnants were obtained from 27 patients subjected to CABG and without venous pathology in their medical history. All patients had chronic angina pectoris and were taking medication, consisting of \( \beta \)-adrenoceptor blockers, long acting nitrates, calcium antagonists, lipid lowering drugs, ACE-inhibitors and diuretics, in various combinations. Written informed consent was received from all patients, and the study was approved by the Ethics Committee of the Academic Medical Center, according to the principles outlined in the Declaration of Helsinki.

In order to standardize the experimental protocol for this material, more or less damaged by the surgical procedure, all preparations were stored in University of Wisconsin solution at 4°C for 2 days and the remaining endothelium was removed by rubbing of the intimal surface with a wooden rod. Each piece of SV was cut into rings of approximately 5-mm length, which were mounted between two L-shaped stainless steel hooks, in 8 mL organ baths filled with oxygenated Krebs-Henseleit solution of 37°C (pH 7.4). Each preparation was fixed, via a silk thread, to an isometric force transducer (A.D. Instruments, Castle Hill, Australia) and force was recorded via a MacLab/8 computer system (A.D. Instruments, Castle Hill, Australia). The preparations were subjected to a pre-tension of 40 mN, which was maintained throughout the experiment. Appropriate controls were run simultaneously in different ring preparations obtained from the same vascular segments.

Experimental protocol

Potassium-induced contractions. After an equilibration period of 60 minutes the vascular rings were primed and tested for viability, by exposing them thrice to an isotonic potassium chloride solution (KCl) of 123.8 mM. The responses induced by the third potassium challenge were taken as maximal (100%). Preparations showing less than 10 mN contraction in response to KCl, were discarded. One hour after the third KCl-induced contraction, the effect of the CA on potassium-induced depolarization was established for verapamil (1 nM- 0.1 mM) or mibefradil (1 nM- 0.1 mM) as shown in Figure 1. In separate experiments this effect was studied in the presence of phentolamine (10 \( \mu \)M).

Noradrenaline-induced contractions. After the priming procedure SV preparations were exposed to cumulative concentrations of noradrenaline (NA) (1 nM- 0.1 mM) to
construct the concentration-response curves. This was repeated after 60 minutes in the presence of the CA (Figure 1).

**Potassium-channels.** After the first exposure to noradrenaline the SV preparations were either incubated with the K\textsubscript{ATP}-potassium-channel blocker glibenclamide (30 mM) or the K\textsubscript{Ca}-channel blocker charybdotoxin 0.1 \textmu M (or vehicle) 60 minutes prior to the second exposure to noradrenaline in the presence or absence of mibefradil.

**Drugs used**
Acetylcholine chloride, glibenclamide, phentolamine hydrochloride, phenylephrine hydrochloride and verapamil hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.); mibefradil (Ro 40-5967) from Hoffman-LaRoche (Basel, Switzerland); noradrenaline bitartrate from Hoechst (Amsterdam, the Netherlands); propranolol hydrochloride from Research Biochemicals International (Natick, U.S.A.); ascorbic acid from Merck (Darmstadt, Germany); charybdotoxin from Bachem (Bubendorf, Switzerland). All drugs were dissolved in distilled water, except noradrenaline and glibenclamide. Noradrenaline was dissolved in a 0.1 mg/mL ascorbic acid containing solution. Glibenclamide was dissolved in 100% DMSO to a stock solution of 80 mM and subsequently diluted with 100% DMSO to the concentrations 8 mM and 0.8 mM, after which they were added to the organ bath. The vehicle did not affect the results as studied in separate control experiments (data not shown).

**Solutions used**
The Krebs-Henseleit solution used for the experiments had the following composition (mM): NaCl 118.0; KCl 4.7; NaHCO\textsubscript{3} 25.0; MgSO\textsubscript{4} 1.2; CaCl\textsubscript{2} 2.5; KH\textsubscript{2}PO\textsubscript{4} 1.1; and glucose 5.6. For the experiments with noradrenaline, propranolol (1\mu M) and ascorbic acid (0.1 mg/mL) were added.
The KCl solution had the same composition as the Krebs-Henseleit solution used, except for the NaCl (118 mM) which had been replaced by an equimolar amount of KCl corresponding to a concentration of 123.8 mM.
The composition of the University of Wisconsin solution (ViaSpan, Du Pont, Wilmington, DE, U.S.A.) has been described previously.\textsuperscript{11}
Figure 1: A graphical representation of calcium and chloride exposure effects on force production. The upper figure shows calcium-induced (upper figure, lower figure, chloride-induced) contractions in human saphenous vein. CA = calcium, P = potassium, K = potassium, CI = chloride, NA = sodium (123.3 M).

Legend:
- \( \text{KCl} \) solution (123.3 M).
- \( \text{Ca}^{2+} \) solution.
- \( \text{Cl}^{-} \) solution.
- \( \text{Na}^{+} \) solution.

Force (mN)
Statistical analysis

The data were expressed as means ± S.E.M. for $n$ observations. The concentration-response curves for the compounds investigated were analysed by using a computer program (Graph Pad, Institute for Scientific Informatics, San Diego, CA, U.S.A.). The pD2-value [-log effective concentration (molar) that produces 50 % of the maximal effect (IC50)], as well as the maximal effect (E_{max}) were thus obtained from the non-linear regression curve fit analysis for the individual experiments. The statistical significance of the differences was analysed by means of two-sided Student’s t-test for unpaired data. Values of P of less than 0.05 were considered significant.

Results

Functional endothelium was absent after the denudation procedure as confirmed by the lack of a vasodilator effect of 1 µM acetylcholine after precontraction with 0.3 mM phenylephrine (data not shown). In 30% of the preparations spontaneous rhythmic contractions were observed which attenuated after application of either one of the two CA (1 µM). The addition of the CA, phentolamine or the potassium channel blockers to the organ bath did not affect the basal tension of the SV preparations.

![Figure 2](image)

Figure 2: The effect of incubation of isolated human saphenous vein preparations with verapamil (O) ($n = 8$) or mibebradil (●) ($n = 7$) on KCl-induced contractions, depicted as concentration-response curves. The KCl-induced contractions in the presence of the CA are represented as % of the responses to KCl, without the CA. Data are expressed as means ± S.E.M. *P < .0001 compared between the two calcium antagonists, as analysed using a 2-sided Student’s t-test.
Endothelium-denuded and in UW preserved isolated human SV preparations demonstrated a 27.3 ± 4.3 mN contraction in response to KCl, which remained stable throughout the experiments.

The incubation of the vein preparations with verapamil or mibefradil caused a concentration-dependent inhibition of KCl-induced contractions as shown in Figure 2. Mibefradil was less potent and less effective than verapamil, in reducing the contractions to KCl, in isolated saphenous vein (Table 1). To exclude an effect of endogenous noradrenaline, released from adrenergic nerve endings in the tissue during depolarisation, the experiments were repeated in the presence of the non-selective α-adrenoceptor blocker phentolamine (10 μM). The addition of phentolamine in the current experiments did not influence the actions of the CA in inhibiting contractions to potassium (Table 1).

Table 1: Effect of mibefradil and verapamil on KCl-, and noradrenaline-induced contractions.

<table>
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<th></th>
<th>n</th>
<th>pD₂</th>
<th>P</th>
<th>E_max</th>
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<td>KCl</td>
<td>M</td>
<td>7</td>
<td>5.4 ± 0.2</td>
<td>.02*</td>
<td>63.3 ± 4.6</td>
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<tr>
<td></td>
<td>V</td>
<td>8</td>
<td>5.8 ± 0.1</td>
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<td>81.2 ± 3.5</td>
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<td></td>
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<td>5.4 ± 0.2</td>
<td>.09#</td>
<td>61.6 ± 3.3</td>
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<tr>
<td></td>
<td>V + Phent</td>
<td>5</td>
<td>6.0 ± 0.1</td>
<td>.03*</td>
<td>75.2 ± 10.5</td>
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<tr>
<td>NA</td>
<td>Ctrl</td>
<td>13</td>
<td>7.1 ± 0.1</td>
<td></td>
<td>95.7 ± 3.6</td>
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<tr>
<td></td>
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<td>62.8 ± 5.8</td>
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<tr>
<td></td>
<td>V</td>
<td>5</td>
<td>6.2 ± 0.2</td>
<td>.0002†</td>
<td>61.0 ± 11.5</td>
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<tr>
<td></td>
<td>M + Gli</td>
<td>5</td>
<td>6.4 ± 0.1</td>
<td>.04‡</td>
<td>58.5 ± 7.5</td>
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<tr>
<td></td>
<td>M + Char</td>
<td>5</td>
<td>6.9 ± 0.1</td>
<td>.07‡</td>
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The effect of verapamil (V) and mibefradil (M) on potassium-induced contractions (KCl) (with/without phentolamine (Phent)). The effect of noradrenaline (NA) in the absence (control (Ctrl)) and presence of mibefradil or verapamil, with/without potassium channel blockers (glibenclamide (Gli) and charybdotoxin (Char)).

Data are expressed as means ± S.E.M. *: verapamil vs mibefradil inhibiting KCl-induced contractions, #: KCl-induced contractions inhibited by calcium antagonists with vs without phentolamine, †: inhibition of NA-induced contractions by calcium antagonists vs control, ‡: NA-induced contractions inhibited by mibefradil with vs without potassium channel blocker. $E_{max}= $ maximal inhibition of KCl-induced contractions, and the maximal response to noradrenaline (as % of the maximal response induced by KCl), $pD_2= -\log $ effective concentration (molar) that produces 50% of the maximal effect ($EC_{50}$).
Figure 3: The inhibition exerted by (A) verapamil (n = 5) (1 μM ● and 10 μM ○) or (B) mibebradil (n = 13) (1 μM ● and 10 μM ○), upon noradrenaline-induced contractions (▲)(n = 13). Data are expressed as means ± S.E.M. *P < .05 for the response of noradrenaline in the presence of calcium antagonist compared to control, as analysed using a 2-sided Student’s t-test.
exclude the β-adrenoceptor-mediated effects of noradrenaline. Noradrenaline induced a maximal absolute contraction of 18.8 ± 1.8 mN in the isolated SV preparations. The contraction mediated by cumulative concentrations of noradrenaline was inhibited to the same extent, and in a concentration-dependent manner, after incubation of the vein rings with either mibefradil or verapamil (Figure 3). The CA caused a decrease in the potency of noradrenaline, as shown by a rightward shift, and reduced the efficacy, as reflected by a downward shift of the concentration response curve (Figure 3, Table 1). It has been submitted that mibefradil is a K<sub>ATP</sub>-channel opener in the myocardium. Under the condition of alpha-adrenoceptor stimulation this property of mibefradil on potassium channels might play a role in its vasodilatory effect. However, the inhibition of NA-induced contractions by mibefradil was insensitive to either K<sub>ATP</sub>-channel blockade by glibenclamide or K<sub>Ca</sub>-channel blockade by charybdotoxin (Figure 4, Table 1).

Discussion

In the present study, the influence of verapamil upon receptor-, and non-receptor-mediated contractions was investigated, and compared to mibefradil. In the pathogenesis of graft spasm both mechanisms of VSMC contraction are thought to be
involved. For the investigation of receptor-dependent responses, noradrenaline was selected. Catecholamines are circulating in high concentrations per- and postoperative CABG. Furthermore, \( \alpha_2 \)-adrenoceptors play a role in cold-induced vasospasm and in human SV noradrenaline activates a combination of postjunctional \( \alpha_1 \) - and \( \alpha_2 \)-adrenoceptors.\(^{12,13}\)

Mibefradil with its particular pharmacological profile appeared to be a promising compound in the treatment of patients that underwent cardiothoracic surgery, especially because of the lack of negative inotropic activity compared to other CA. Despite the fact that mibefradil is no longer available for clinical use, in this study it was applied as an innovative pharmacological tool to analyse the mechanism underlying vasospasm and the mechanisms of action of CA in preventing spasm.

In the SV preparations incubated with the CA, verapamil was demonstrated to be a more potent and effective inhibitor of KCl-induced contractions than mibefradil. The shown difference in potency between the two CA is likely to be due to their affinities for L-type calcium channels. The threshold potential for opening of T-type calcium channels is lower than that of L-type channels, and T-type calcium channels are rapidly inactivated. Therefore, under the conditions of prolonged KCl-induced membrane depolarization only the L-type calcium channels are activated.\(^1\) The difference in efficacy between the two compounds has to be explained by a difference in modulating the probability that the channel is open resulting from binding at the L-type calcium channel. Verapamil and mibefradil bind at different sites of the L-type calcium channel, and this may explain a higher blocking activity of verapamil, as found in our present experiments.\(^8\)

In the present study neither verapamil nor mibefradil inhibited the responses to KCl completely. An interfering role for release of noradrenaline from adrenergic nerve endings in the tissue during potassium-induced depolarization, resulting in a contraction less sensitive to calcium channel blockade, could not be confirmed. From this observation we also conclude that N-type calcium channels, present on the nerve endings, are probably functionally irrelevant in human SV preparations. For both verapamil and mibefradil it has been described that these CA are more effective in reducing contractions when administered after instead of before the contraction to KCl.\(^{14}\) The contribution of intra-, and extracellular \( \text{Ca}^{2+} \) may differ for the initiation and the maintenance phase of VSMC contraction and the binding affinity of certain CA for the calcium channel increases during depolarisation.\(^{14,15}\) Therefore, the effect of CA may vary, depending on the moment of application.

Contractions of VSMC evoked by \( \alpha \)-adrenergic stimulation are the result of the release of calcium from intracellular stores, the increase in sensitivity of the contractile
apparatus to calcium and the depolarization resulting from the increase in intracellular calcium. In the present study the two CA (10 μM) inhibited the noradrenaline-induced vasoconstrictor responses equally well. The fact that verapamil inhibited responses to potassium more effectively than noradrenaline-induced constrictor effects is probably due to the higher number of L-type calcium channels activated during KCl-depolarisation compared to α-adrenergic activation. The rightward shift of the noradrenaline concentration response curve might indicate an antagonism at the level of the α-adrenoceptor by both CA. This effect has been described previously for verapamil, but thus far not for mibefradil.

In the condition of α-adrenergic activation the depolarisation is expected to be less pronounced than during exposure to 123.8 mM KCl. Therefore, under the condition of α-receptor stimulation the activity of potassium channels is expected to be relevant. Since mibefradil displays a glibenclamide-sensitive effect in the myocardium, the present study evaluated the effect of mibefradil on K\textsubscript{ATP}, and K\textsubscript{Ca}-channels during NA-induced contractions. In human SV mibefradil's action on responses to noradrenaline was independent of glibenclamide-sensitive or charybdotoxin-sensitive mechanisms.

Mibefradil has been demonstrated to stimulate the release of nitric oxide from the endothelium and to facilitate its effect in VSMC in various arteries. Some other CA exhibit a similar effect. However, in a previous study we have shown that the endothelial function of human SV preparations is impaired because of injury during the surgical procedure. Accordingly, this effect of the CA does probably not play a significant role, with respect to the efficacy of CA in the treatment or prevention of venous graft spasm.

In summary, after incubation of isolated, endothelium-denuded human SV preparations, verapamil was demonstrated to be more potent and effective in inhibiting KCl-induced contractions, but equipotent and effective in reducing responses to noradrenaline when compared to mibefradil. Potassium channel opening properties of mibefradil in isolated veins could not be confirmed. The functional role of both N-type as well as T-type calcium channels blockade by mibefradil in isolated SV preparations proved negligible.

Under the conditions investigated the mode of action of CA in the suppression of vasospasm in human SV grafts can be explained by two pharmacological properties of the compounds: first the L-type calcium channel blocking effect and second the antagonism at the level of the α-adrenoceptors. Since both mechanisms are thought to be involved in the pathogenesis of graft spasm the current findings explain the effectiveness of prevention of graft spasm with CA. Any advantage of additional T-, and N-type calcium channel blockade of mibefradil could not be confirmed in isolated SV preparations. As demonstrated earlier by Roubos et al. the topical and intraluminal
application of a solution of verapamil in combination with glyceryl trinitrate during the
harvest of the saphenous vein improved endothelial function and prevented vein spasm
sufficiently. Also in this study verapamil, demonstrated to be a potent dilator of SV and
it can be recommended to apply this CA in a clinical setting to prevent spasm of
saphenous vein.

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References


