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

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Citation for published version (APA):
INTRODUCTION

1.1. Historical considerations

Human kind has suffered from "exertion-induced chest pain" and high blood pressure for centuries and, not surprisingly, the ancient Chinese physicians already practiced pharmacological intervention in these conditions. They used extracts from the roots of the plants *Salvia miltiorrhiza* and *Stephania tetrandra*, providing tanshinone IIA and tetrandrine as active compounds. When administered to patients, both compounds lower blood pressure, relieve anginal symptoms and exert negative inotropic activity on the heart. Tetrandrine is also capable of terminating acute episodes of paroxysmal supra-ventricular tachycardia. Further research classified these two compounds as calcium antagonists (CA) [1,2]. Although these two CA probably were the first to be used in history, Godfraind and Kaba (1969) were the first to describe an inhibitory action of cinnarizine and chlorpromazine on contractions induced by calcium and adrenaline in depolarized arterial smooth muscle [3]. Prenylamine and iproveratriol (later verapamil) were the first compounds to be denoted as calcium antagonists. Albrecht Fleckenstein and colleagues described in 1971 how they, merely by chance, found that calcium reversed the effects of prenylamine and iproveratriol in the myocardium [4]. Subsequently, nifedipine and diltiazem were recognized as clinically useful compounds. Only in the 1980's the development of calcium antagonists (CA) was taken up seriously and in a short time many new CA were synthesized. At present, the arsenal of available CA for clinical use comprises some 40 compounds and the list is still growing.

1.2. Calcium, calcium channels and vascular smooth muscle

When vascular smooth muscle cells (VSMC) are in the resting state, the calcium concentration within the VSMC is much lower (<0.1 μM) than in the extracellular fluid. (1-2 mM). The resting membrane potential of VSMC is in the range of -45 to -70 mV. However, although there exist both a concentration and an electrical
gradient favouring Ca$^{2+}$-entry into the cell, the rate of entry is very low. Excitable calcium channels are closed in the resting state and passive diffusion across the plasmalemma is negligible, because calcium ions are highly water-soluble [5].

In vivo, VSMC are not in the resting state, since they are subject to basal vasomotor tone. Vasomotor tone is the sum of the responses to a variety of both vasoconstrictor and vasodilator signals: 1. Neurotransmitters from sympathetic perivascular nerves (e.g. noradrenaline, ATP, neuropeptide Y); 2. Endothelial factors (e.g. endothelin-I, nitric oxide, prostaglandins); 3. Vasoactive substances in the blood stream (e.g. noradrenaline, adrenaline, vasopressin, angiotensin-II, bradykinin, endothelin, vasoactive intestinal peptide, atrial natriuretic factor); 4. Physicochemical factors of the blood (oxygen and carbon dioxide tension, pH, temperature) and 5. Physical factors (intraluminal pressure and stretch) [5].

In this partially activated state the VSMC can contract rapidly when a sudden rise in cytosolic calcium occurs. A calcium influx, which is sufficiently large to provoke contraction takes place through voltage-dependent calcium channels (VDCC) and probably also through receptor-operated calcium channels. Since investigations described in the present thesis are primarily concerned with the interaction between CA and VDCC, we will emphasize the VDCC.

At least six types of VDCC are known, but only three of them are thought to play a role in the vasculature: L-, T- and N-type calcium channels. The characteristics of these channels are listed in Table 1.

The composition of the VDCC is globally the same for all types of these calcium channels and is depicted in Fig.1. The VDCC consists of a complex of several subunits: the $\alpha_1$-subunit and several auxiliary subunits. The $\alpha_1$-subunit, structurally resembling potassium and sodium channels, consists of six $\alpha$-helical membrane-spanning segments and a single pore-lining region in each of the four motifs. In each motif, the fourth segment is believed to act as the voltage sensor, whereas a, throughout the evolution, consistently preserved region between segments five and six (P-region) is regarded to line the ion permeation pathway. The $\alpha_1$-subunit also contains the binding sites for all known pharmacological modulators of the channel. Several subtypes of this subunit are known and each is representative of a different type of calcium channel [6].

The $\alpha_2$-$\delta$-subunit, a largely, but not completely, glycosylated protein, enhances calcium-flux when co-expressed with the $\alpha_1$-subunit. Another important part for the function of the channel is the $\beta$-subunit. The $\beta$-subunit can increase the level of channel expression and modulate the rate of channel closure. Several different genes encoding for this intracellular subunit have been identified.
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The γ-subunit has been found only in channels of skeletal muscle, but its function so far remains unknown [6]. In Table 1, the various types of α1-subunits for each type of calcium channel are listed.

Table 1. Overview of the composition and characteristics of the three main types of voltage-dependent calcium channels and their respective blockers. Abbreviations: mV = millivolts; pS = picoSiemens; PKA = protein kinase A.

<table>
<thead>
<tr>
<th></th>
<th>L-type</th>
<th>N-type</th>
<th>T-type</th>
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<tbody>
<tr>
<td>α1-subunit</td>
<td>α1S, α1C, α1D</td>
<td>α1B</td>
<td>α1G, α1H</td>
</tr>
<tr>
<td>Activation range</td>
<td>-20mV to pos.</td>
<td>-20mV to pos.</td>
<td>-70 to -30mV</td>
</tr>
<tr>
<td>Blockers</td>
<td>Nifedipine,</td>
<td>Mibefradil, Ni2+</td>
<td>ω-conotoxin</td>
</tr>
<tr>
<td></td>
<td>Verapamil,</td>
<td>flunarizine</td>
<td>GV1A</td>
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<td></td>
<td>Diltiazem</td>
<td></td>
<td></td>
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<tr>
<td>Conduction regulation</td>
<td>25 pS</td>
<td>20 pS</td>
<td>5-8 pS</td>
</tr>
<tr>
<td></td>
<td>Slow inactivation, PKA-modulated</td>
<td>Moderate rate of inactivation</td>
<td>Fast inactivation</td>
</tr>
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L-type calcium channels (LCC) belong to the group of high-voltage activated calcium channels. They have been demonstrated to occur in skeletal muscle, smooth muscle cells and in cardiac and neuroendocrine tissue. Activation of the LCC leads to massive calcium influx with subsequent muscular contraction, reflecting the excitation-contraction coupling process. In the VSMC two ways of opening the channel are known: electromechanical and pharmacomechanical coupling. Electromechanical coupling involves membrane depolarization, leading to an increase in the membrane potential. When depolarization is sufficiently strong, the threshold of -40 mV is passed and the VDCC will open, resulting in a massive influx of calcium and subsequently a contractile response [6].

Pharmacomechanical coupling involves either one of three processes: phospholipase C (PLC)-coupled receptor activation, receptor activation not coupled to PLC and voltage-independent activation of VDCC. The majority of excitatory agonists (such as noradrenaline, angiotensin II and endothelin) bind to PLC-coupled receptors. The activated PLC catalyzes the formation of inositol triphosphate (IP3) from the phospholipid PIP2. IP3 diffuses from the membrane to the cytosol where it induces calcium release from the sarcoplasmic reticulum (SR). The elevated levels of calcium and IP3 activate a protein kinase C (PKC) which moves from the cytosol to the cell membrane where it is further activated by diacylglycerol (DAG).
Fig.1. Overview of some key structural properties of voltage-gated calcium channels. A: Oligomeric architecture of typical calcium channel includes the pore-forming $\alpha_1$-subunit, and $\alpha_2$-$\delta$- and $\beta$-subunits. B: Putative structure of $\alpha_1$-subunit, including four homologous domains, each containing six membrane-spanning helices and a pore-forming segment (P-region). The P-regions are thought to act as staves of a barrel when the pore is viewed from above. Carboxylated side chains of conserved glutamate residues, one in each of the four P-regions are believed to form a site for calcium selectivity (Acc. to Tsien RW et al. J Cardiovasc Pharmacol (1996); 27(Suppl.A):S4-S10.

PKC induces a slowly developing contraction involving phosphorylation of the VDCC with subsequent opening of the L-channel. Other mechanisms by which this PKC enhances contraction are: 1. Increasing the affinity of the myofilaments to calcium by phosphorylating myosin light chain kinase (MLCK) and or the myosin light chain (MLC$_{20}$) itself; and 2. Increasing intracellular pH to a value of 8, which is optimal for the interaction between IP$_3$ and the SR. The increase of cytosolic calcium released from the SR as such can depolarize the cell membrane and thus trigger the opening of the VDCC [6].

The $\alpha_{2A}$-adrenoreceptor is not coupled to PLC. Activation of this receptor, located in arterioles and veins, leads to a rise in cytosolic calcium probably via non-selective cation channels. This increase in cytosolic calcium probably induces a process known as calcium-induced calcium release from the SR; together they increase the calcium concentration sufficiently to cause membrane depolarization.
Bay K 8644 is a calcium channel agonist which is able to activate VDCC directly by interacting with the transmembrane segment IVS6 of the $\alpha_1$-subunit [7].

1.3. Contraction

After the opening of the L-type calcium channel, the suddenly abundant cytosolic calcium forms a complex with calmodulin (four Ca$^{2+}$-ions on 1 calmodulin molecule). This complex binds to an inactive MLCK to form an active holoenzymatic complex that catalyzes the transfer of a phosphate group from ATP-Mg$^{2+}$ to a specific serine residue of the regulatory MLC$_{20}$. This phosphorylation permits activation of the myosin-ATPase by actin, leading to enhanced ATP hydrolysis and, thus, to an increase in the crossbridge cycling rate and, consequently, to the development of isometric tension [5]. Fig.2. depicts an overview of the calcium fluxes in an activated vascular smooth muscle cell (VSMC).

The LCC are the main target for almost all known CA.

The N-type calcium channel (NCC) is another member of the group of high-voltage activated calcium channels. This channel is found in (N)euronal tissue, hence its nomenclature. It is an important channel for the vasculature, since it regulates the release of neurotransmitter-containing vesicles from the perivascular sympathetic nerves [8].

The only known low-voltage activated calcium channel is the T-type calcium channel (TCC). Its function is still much debated. Evidence accumulates concerning its involvement in carotid restenosis and cardiac arrhythmias, but hard evidence is lacking because the structure of the channel was not known [9-11].

Only very recently the $\alpha_1$-subunit of a TCC located in the brain was cloned by Perez-Reyes et al. [12]. Expression of this $\alpha_{1G}$-subunit was highest in the brain (amygdala, thalamus and subthalamic nuclei) and less so in the heart. Prolonged exposure revealed expression in lungs, kidney and placenta. Another problem in the research concerning the TCC is the lack of specific antagonists. Several moderately selective antagonists are known, like mibebradil, but its effectiveness in simultaneously blocking the LCC as well at therapeutic concentrations limits its use as a tool for a sophisticated analysis of the T-type calcium channel.
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Fig. 2. Cellular events triggered by VSMC activation. For clarity, the diagram does not include protein kinase C (PKC) activation by diacylglycerol (DAG) and Ca\(^{2+}\) homeostatic mechanisms, which also operate at rest and which may be activated by any of several protein kinases and/or calmodulin. The sarcoplasmic reticulum (SR) is illustrated with separated ryanodine- and IP\(_3\)-accessed compartments, with a partial connection activated by GTP. Receptor operated channels (ROCC) and mechanosensitive calcium channels (not illustrated) may be nonselective cationic calcium channels (activated by intracellular calcium). Ca\(^{2+}\) influx through these channels may induce membrane depolarization, activated voltage-operated calcium channels (VOCC), and participate in refilling (not shown). Abbreviations: G = G-protein; SMOCC = second messenger operated channel; DOCC = depletion-operated calcium channel; CIF = calcium influx factor (Reprinted from Orallo F. Pharmacol Ther (1996); 69(3):153-171.

1.4. Classification of calcium antagonists

Since many agents have been classified as calcium antagonists and there is a broad spectrum of structural diversity among these agents, a classification remains
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Several classifications have been proposed, on the basis of chemical structure, receptor-binding specificity or tissue selectivity. In 1996 Triggle described the most frequently used mode of classification, based on the chemical structures. This classification involves the type of VDCC, the specific site of interaction and the modulation of this interaction. The most important groups of CA used in the treatment of cardiovascular disease are the 1,4-dihydropyridines (1,4-DHP), phenylalkylamines (PAA) and benzothiazepines (BTZ) [13].

The 1,4-DHP-group is the most widely represented and investigated group of CA. These agents specifically interact with the 1,4-DHP-site on the LCC. Since the introduction of the first 1,4-DHP, nifedipine, numerous new compounds have been synthesized, and most of them display a more favourable profile than nifedipine. Where nifedipine causes a significant negative inotropic effect and reflex tachycardia, the newer 1,4-DHP seem to be devoid of such unwanted side-effects [14-16]. Because of their higher lipophilicity and/or ionizable side chain and/or increased bulkiness of the side chain, their onset of action is much slower and the duration of action is much longer [17]. This improved kinetic profile makes once-daily dosing possible, thus improving patient compliance. Since for nifedipine it has been shown that the rate of infusion largely determines whether reflex tachycardia occurred or not [18], a special system known as GastroIntestinal Therapeutic System (GITS) was developed [19]. This is a slow-release system giving nifedipine a kinetic profile resembling that of the newer, more lipophilic CA. For other CA (like verapamil and diltiazem) systems like GITS also have been devised [20-22]. Although nifedipine already shows a certain degree of vascular selectivity, newer 1,4-DHP are even more vasoselective, thereby minimizing the negative inotropic effects. This development is very important, since patients with hypertension are treated for life and many of these patients have a compromised cardiac function or will have so in the future. Examples of the newer 1,4-DHP are amlodipine, barnidipine, cilnidipine, felodipine, lacidipine, manidipine and lercanidipine. The major indications for prescription of a 1,4-DHP are essential hypertension and angina pectoris [23].

The PAA are another type of CA with verapamil as their first and main representative. PAA specifically interact with the PAA-site on the LCC and display a clinical profile different from that of the 1,4-DHP. Next to a moderate vasodilator effect and a slight negative inotropic action, PAA decrease heart rate by directly interfering with atroventricular node conduction, whereas a depressant effect on the sinus node also occurs. This negative chronotropic effect has proven beneficial in the treatment of supraventricular tachyarrhythmias, in particular atrial fibrillation [24]. So far, the very few newly developed CA of this class (e.g. gallopamil, devapamil, (-)-D888) do not seem to offer any additional advantage over verapamil.
The third class of CA, the benzothiazepines, is clinically represented by diltiazem. As for verapamil, the development of new analogues is slow and therefore only a few new compounds are under investigation (clentiazem, DTZ323 and TA3090). As for the previous two classes of CA, BTZ possess a distinct binding site on the LCC. The indication for the prescription of diltiazem is predominantly stable angina pectoris [25].

In 1986 a novel phenylalkylamine-derived CA was introduced by Roche Pharmaceuticals, Ro 40-5967. The compound possesses a verapamil-like chemical structure. It interacts in a dually competitive manner at the phenylalkylamine and indolizinesulfone sites of the LCC [26]. However, in numerous electrophysiologic studies in Xenopus oocytes and isolated VSMC the compound’s affinity for the TCC appeared to be much higher than for the LCC [27,28]. Now known as mibefradil, it is the first CA which has a higher affinity for the T-type calcium channels than for the LCC.

1.5. Haemodynamic actions

1.5.1. Antihypertensive activity

Vasodilation is a major property of all CA, underlying most of their therapeutic actions. Vasodilation occurs predominantly at the level of the resistance vessels (precapillary arterioles), thus causing a reduction in elevated peripheral resistance, as in essential and other forms of hypertension [29]. In therapeutic doses, the CA exert no primary dilator effect on the venous system, thus explaining the absence of orthostatic hypotension during treatment with these agents [29-31], in spite of their potent antihypertensive activity.

The fall in blood pressure and vascular resistance trigger a sinoaortic baroreflex-mediated rise in sympathetic activity, as reflected by the transient tachycardia associated with nifedipine and other dihydropyridine CA [29,31]. The direct depressant cardiac actions of verapamil and diltiazem, in addition to their interference with the baroreflex mechanism, prevent a rise in heart rate and cardiac output. As a result of a long-term adaptation process, heart rate and cardiac output return to values close to (DHP) or slightly below (verapamil, diltiazem) control levels [29,31].

Vasodilation occurs especially in the skeletal muscle and coronary vascular beds [29,31]. The gastrointestinal, cerebral and renal vascular beds are also dilated, whereas the skin is only slightly affected, although facial flushes may occur as an adverse reaction to dihydropyridine CA.
Vasodilation in the coronary system, in particular the relaxation of coronary spasm, is a major component of the anti-ischemic activity of CA in coronary heart disease. A mild natriuretic effect, probably at the renal distal tubular level may explain why the CA, although potent vasodilators, do not cause fluid retention. Peripheral (ankle) edema, a well-known side effect of the dihydropyridine CA, does not reflect systemic fluid retention, but rather a direct effect on the microcirculation, and possibly also on the lymphatic circulation [30].

With long-term use, CA may induce a regression of myocardial and vascular hypertrophy [32]. Several attempts have been made to establish a causative relationship between calcium metabolism/fluxes and the pathogenesis of hypertension, but no such relationship has so far been demonstrated convincingly.

1.5.2. Anti-anginal activity

The imbalance between myocardial oxygen supply and demand, which underlies myocardial ischaemia and its sequelae such as angina pectoris, may be improved by CA by a reduction in peripheral vascular resistance and, in consequence, a reduction in cardiac afterload and left ventricular wall tension; together with coronary vasodilation these mechanisms will reduce myocardial oxygen consumption [33,34]. A reduction in heart rate, as induced by verapamil or diltiazem, further contributes to the anti-anginal activity of these two compounds, by reducing the oxygen consumption of the myocardium. In addition the relief of coronary spasm and a reduction in coronary resistance will improve the supply of oxygen to the myocardium. The DHP, as previously mentioned, either cause reflex tachycardia (nifedipine in the non-retarded formulation) or leave the heart rate unchanged.

At the cellular level, most CA are thought to preserve ATP-levels in the ischaemic heart, as demonstrated in animal and biochemical experiments. However, there is no evidence that this cellular anti-ischaemic mechanism is of clinical importance in the treatment of angina [35].

1.5.3. Secondary prevention after acute coronary syndromes

Verapamil is the only CA for which a clear protective effect has been shown in patients who have suffered an acute coronary syndrome. Increased survival and a lower re-infarction rate were found in the 2nd Danish Verapamil Infarction Trial (DAVIT II) study among patients treated for 12-18 months after a myocardial infarction (verapamil vs placebo) [36,37]. In a smaller study with diltiazem, the Diltiazem Multicenter Postinfarction Research Trial, a favourable trend was seen following a myocardial infarction in patients without pulmonary congestion after long-term treatment with diltiazem (compared with placebo) [38].
Nifedipine has no protective effect in patients who have survived an acute coronary syndrome. In patients who had experienced unstable angina the incidence of subsequent myocardial infarction was higher in those treated with nifedipine, compared with placebo. This negative effect of nifedipine was suppressed by simultaneous treatment with a β-blocker (Holland Interuniversity Nifedipine Trial: HINT) [39].

The reason for the cardioprotective effect of verapamil in the DAVIT II study has not been determined. It seems likely, however, that the reduction in heart rate may have been an important beneficial factor. Conversely, the reflex tachycardia provoked by nifedipine may explain the unfavourable effects to this CA in the HINT study [39]. The favourable effect of adding a β-blocker in this study was associated with the suppression of the nifedipine-induced reflex tachycardia. Taken together the suppression of tachycardia by verapamil is likely to have been an important component of the protective action of this CA in the DAVIT-II trial.

1.5.4. Anti-arrhythmic activity

Verapamil and sometimes diltiazem may be used as antiarythmics in the treatment of supraventricular tachy-arrhythmias. The anti-arrhythmic activity is based upon an impairment of the electrical activity in the cardiac nodal tissues, causing impaired A-V conduction and a reduction in heart rate. In contrast, the dihydopyridine CA display no useful anti-arrhythmic activity.

In clinical practice verapamil may be used both intravenously and via the oral route.

1.6. Calcium antagonists and left ventricular hypertrophy

Left ventricular hypertrophy (LVH) is considered as an important, virtually independent risk factor in hypertensives, already recognised in the Framingham Study [40]. It is therefore an important issue whether treatment with antihypertensive drugs will cause regression of LVH.

Numerous animal experiments have indicated that CA may cause regression of the LVH associated with hypertension. In clinical studies this issue has been followed up and most of these investigations have shown that various types of CA indeed cause regression of LVH on long-term treatment [32,41,42]. Attempts have been made to compare the efficacy of different types of antihypertensives in this respect [43-46]. The impression is obtained that ACE-inhibitors and CA are the most efficacious antihypertensive drugs with respect to the regression of LVH in hypertensives, and probably more effective than β-blockers or diuretics [46]. The studies so far performed do not allow a quantitative and definite conclusion with respect to the
different efficacies of various types of drugs, also because of methodological difficulties in the quantitative determination of LVH in patients [46]. Trials designed in such a manner that quantitative comparisons between various drugs can be made are on the way.

Taken together there are sound reasons to assume that long-term antihypertensive treatment (at least for several months) with CA will cause significant and relevant reduction of LVH. For review on this issue see [32,46,47].

In studies with isolated vascular smooth muscle cells in isolated vessel preparations CA have been shown to impair vascular smooth muscle proliferation [48]. This potentially beneficial effect may also occur in the treatment of hypertensive patients. Because of methodological difficulties such an effect has so far not been convincingly demonstrated in human hypertensives treated with CA.

1.7. Calcium antagonists and atherosclerosis

In vitro and animal studies have strongly suggested that CA may impair lipid accumulation in the aorta. CA have been demonstrated to impair a variety of processes which underly atherosclerotic plaque formation, such as cholesterol deposition, cellular proliferation and migration, increased cellular matrix, calcium overload and platelet aggregation [48]. The anti-atherogenic activity of CA is not mediated by a reduction in plasma lipid levels, which are not influenced by CA in therapeutically active doses. The impression is obtained that lipophilic CA such as lacidipine and lercanidipine are the more active anti-atherogenic agents, at least in animal and biochemical experiments. So far it has been very difficult to demonstrate an anti-atherogenic effect of CA in human patients.

Nifedipine was shown to impair the formation of new lesions in the coronary arterial system in patients with ischaemic heart disease [49]. Large, established lesions, however, were not affected by nifedipine treatment, and nifedipine did not protect against acute coronary syndromes in these patients [49].

Comparable findings were obtained for nicardipine in the Montréal Heart Study [50]. In the MIDAS-Trial isradipine and hydrochlorothiazide were compared in mild to moderate hypertensives. Atherosclerotic plaque formation was monitored by means of an echo-doppler procedure. Unfortunately, the data concerning an anti-atherogenic action of isradipine were inconclusive because of methodological difficulties [51].

In the ELSA-study (European Lacidipine Study on Atherosclerosis about 2300 patients were randomised to lacidipine or atenolol [52]. Both the antihypertensive effect and a possible influence on plaque formation in the carotid region are
monitored. The study is ongoing. In the similarly designed VHAS-study verapamil and captopril are compared [53].

In conclusion, the potential anti-atherogenic activity of CA is a most important issue, but it remains to be demonstrated whether it indeed occurs in hypertensive patients when treated with these drugs.

1.8. Calcium antagonists and perioperative hypertension

Perioperative hypertension, predominantly caused by sympathetic stimulation, occurs frequently during thoracic surgery. Usually, this acute form of hypertension is suppressed by using short acting, intravenously administered antihypertensive drugs. Nitroglycerin, sodium nitroprusside, clonidine, urapidil, ketanserin, and also a few short-acting β-blockers or CA may be used for this purpose [54]. Among the various types of CA, only DHP, which are predominantly vasodilator agents with little or no cardiodepressant activity, have been studied in detail in surgical patients in an effort to counteract or prevent peri-operative hypertension. Among the numerous DHP available at present, most of the studies have been limited to nifedipine and nicardipine. A few smaller studies have been performed with isradipine (a newer DHP) and with diltiazem. As a whole, nifedipine and nicardipine are considered useful, short acting DHP-CA in the treatment of perioperative hypertension [54-56].

1.9. Long term safety

The safety of long-term treatment with CA has recently been challenged by three studies, two case-control studies in hypertensives [57,58], and a meta-analysis of 16 studies on nifedipine aiming at secondary prevention subsequent to acute coronary syndromes [59]. From these studies it was concluded that long-term treatment with CA may be associated with a higher risk of cardiovascular morbidity and mortality. It has also been submitted that long-term use of CA may elevate the risk of cancer in elderly populations [60]. From smaller studies it has been concluded that CA would enhance the risk of bleeding in the digestive tract [61], or during surgical interventions [62]. In the case-control studies in hypertension, CA appeared to cause a modest increase in the risk of myocardial infarction while the meta-analysis appeared to show that nifedipine was associated with increased mortality in patients with symptomatic coronary heart disease. However, all three studies have severe methodological flaws.
and their interpretation has been strongly criticized [63-65], with respect to the following issues: erroneous calculations, conclusions drawn too "heavy" on the basis of too meagre statistics, and confounding by indication. Furthermore, it should be realised that the relevance of case control studies is limited, especially if the differences between cases and controls is as modest (increase of risk by not more than 60%) as in the aforementioned cardiovascular studies. As a rule the relevance of case control studies is considered as valid if the difference between cases and controls is at 300%, as encountered for instance in the large retrospective studies on the association between smoking cigarettes and lung cancer in the 1960s.

There is considerable uncertainty about the negative conclusions drawn from the cardiovascular studies, as reflected by the recent United States Food and Drug Administration (FDA) decision not to change the general policy towards the use of CA as antihypertensive agents. Conversely, more and more data are now beginning to emerge which severely challenge the validity and relevance of the aforementioned studies on CA.

In a case control study by Aursnes [66], designed similarly as those already mentioned, CA did not increase the risk of cardiovascular morbidity or mortality, and it even proved to be protective. In a cohort study by Braun [67] in 15502 patients with coronary heart disease the risk ratio of death was not influenced by CA, neither in the positive nor in the negative sense.

In a case control study by Alderman et al. [68] hypertensive patients with a history of at least one major cardiovascular event were compared with correctly matched controls. The rapidly acting, non-retarded nifedipine increased the risk of death (RR = 3.88) when compared with a β-blocker. However, the more recently introduced slow- and long-acting CA significantly reduced the risk of death (RR = 0.76) when compared with a β-blocker. Both in the STONE- and the SYST-EUR-studies [69,70] total mortality was unchanged by CA-treatment, whereas protection against cerebro- and cardiovascular events was obvious. In neither of the two studies an increased risk of cancer or gastrointestinal bleeding was observed. In a recent communication by Lever [71] it was reported that in the well-known WOSCOP trial [72] the survival, cardiovascular morbidity/mortality and incidence of cancer proved uninfluenced by the long-term use of CA. The recently published HOT-study did not show any evidence for noxious effects of felodipine on mortality, MI or cancer [73]. The recently published STOP-2 study demonstrated that CA are as effective as various other antihypertensive drugs (diuretics, β-blockers, ACE-inhibitors) with respect to the lowering of blood pressure and the protection against cerebro- and cardiovascular events. Furthermore this large study did not show any evidence for an increased risk of mortality, MI or cancer [74].
In conclusion, the challenge of the long-term safety of CA is controversial and not convincing. In contrast, more and more solid data are emerging which demonstrate the long-term safety and the beneficial effects of CA. Considerable doubt has been cast upon the safety of rapidly and short acting 1,4-DHP such as non-retarded nifedipine. It seems wise to replace this agent by the slow- and long-acting CA discussed in the forthcoming on the new CA. The validity and safety of these newer CA are subject to critical tests in the ongoing trials.

1.10. New calcium antagonists

1.10.1. General trends
Several new CA have been introduced as potential therapeutic agents in cardiovascular medicine. The majority of these compounds are DHP, although an interesting verapamil derivative (mibefradil) has been developed as well. The disadvantages of nifedipine (negative inotropic activity, short duration of action and reflex tachycardia) stimulated the development of DHP-CA with less negative inotropic activity and a more favourable pharmacokinetic profile. A certain degree of vasoselectivity claimed for these newer compounds implies that they may have less or possibly no depressant action on cardiac contractile force, while the therapeutic efficacy is at least the same as or even better than that of nifedipine. Indeed, several of the newer DHP-CA as well as the verapamil-like CA mibefradil appear to have a certain degree of vascular selectivity, which implies that in therapeutic doses they induce little or no cardiodepressant activity, unlike nifedipine, verapamil or diltiazem.

An even more sophisticated approach has been followed in current attempts to develop CA with selectivity for a particular vascular bed. Furthermore, pharmacokinetic improvements of newer CA have indeed been achieved for several DHP, but also for verapamil, diltiazem and mibefradil. These pharmacokinetic improvements are based upon two different principles:

1. the development of slow release (retarded) preparations of CA which are as such rapidly and short acting;
2. the development of CA with a slow and long duration of action of their own.

1.10.2. Slow release preparations
The non-retarded nifedipine capsules were replaced by a slow-release preparation which has to be administered twice daily in order to obtain an acceptable control of blood pressure in hypertensives, which is associated with less intensive reflex tachycardia when compared with the non-retarded preparation. More recently a more
sophisticated retarded preparation of nifedipine has been introduced as the nifedipine-gastrointestinal therapeutic system (GITS) [75,76]. This preparation is based upon the principle of an ALZET-minipump, which after oral ingestion is compressed via an osmotic mechanism and hence slowly squeezes out the solution of nifedipine with zero-order kinetics into the intestinal lumen. Consequently, a constant, slow release of the active drug (nifedipine) is achieved, thus leading to a slowly developing, long lasting effect, high bioavailability, stable blood levels, as well as a favourable trough to peak ratio [75,76].

Felodipine, isradipine and nicardipine are also available as slow-release preparations, as well as the so-called coat-core formulation of nisoldipine [77]. Similarly both verapamil and diltiazem are now available as slow-release preparations. All of these preparations allow a once daily administration with acceptable or good trough to peak ratios, slow onset of action, and the virtual absence of reflex tachycardia or neuro-endocrine activation.

1.10.3. Slow and long-acting CA

A second possibility to obtain CA with an improved kinetic profile is based upon the use of molecules which as such slowly develop their effects and maintain their action sufficiently long to allow a once daily administration for the treatment of hypertension or angina.

Amlodipine, a DHP, was one of the first examples of such slow and long acting CA. From a chemical point of view, amlodipine is unusual with regard to its side chain, which is ionized at pH = 7.4. This makes the molecule amphipathic: it penetrates well into biological membranes and stays there because of interaction between the ionized side chain and the hydrophilic part of the lipid bilayer [15]. Furthermore the molecule is rather stable and hepatic degradation occurs but slowly [77].

1.10.4. Lipophilic CA

Several newer DHP-CA may be characterized as lipophilic molecules. The lipid solubility of such compounds implies that they readily dissolve in lipid-rich depots, including those within the cell membrane. From these depots they are slowly released to subsequently reach their subsequent receptor, that is the L-type calcium channel. This process, as described by a three-compartment model, is depicted in Fig.3. This phenomenon explains their slow onset of action and, therefore, the virtual absence of reflex tachycardia and sympathetic activation. The persistence of such lipophilic CA within the cell membrane, has been explored in detail for lacidipine and lercanidipine [78-81]. The antihypertensive action of a single dose of lacidipine persists for 24 h and the circadian rhythm of blood pressure is maintained, although at a lower level. Interestingly, heart rate remains unchanged, in spite of the
vasodilator/antihypertensive action of lacidipine, indicating that no reflex tachycardia is triggered. Similar findings have been obtained with lercanidipine, also a lipophilic CA [80,81].

1.11. Vascular selectivity

Amlodipine, barnidipine, felodipine, isradipine, lacidipine, manidipine, nicardipine, and nisoldipine are examples of DHP-CA with a certain degree of vascular selectivity. The stronger effect of these agents on the vascular system than on the heart has been attributed to differences in α₁-subunit or differential expression of the other types of subunits which together form the L-type calcium channels in the various tissues and organs.

![Diagram of vascular selectivity](image)

Fig. 3. Three compartment model of a lipophilic CA (lacidipine) explaining slow onset and long duration of lipophilic CA. (Reprinted from Micheli et al. J Cardiovasc Pharmacol (1991); 17(Suppl.4): S1-S8.

Nisoldipine has been claimed, among the DHP-CA to display the highest degree of vascular selectivity, but this claim is only based upon in vitro experiments with isolated human and animal blood vessels [82]. A few CA have been claimed to display moderate selectivity for a particular, specialized vascular bed. The evidence for such claims is in most cases rather meagre. A certain degree of selectivity for a particular vascular bed is probably best substantiated for the renovascular selectivity of manidipine, which indeed shows most potent dilator effects on renal vessels in intact animals and in vitro, whereas improved renal function in conditions of renal insufficiency have been demonstrated
in animal models [83]. Clinical data which do not contradict the claim of renal selectivity are beginning to emerge [84].

1.12. Mibefradil

Mibefradil has been derived chemically from verapamil, although its molecule contains a few rather unusual substituents. Mibefradil appears to bind to the same (3H)-desmethoxy-verapamil binding sites as verapamil in cardiac membrane homogenates. The haemodynamic profile of mibefradil may be briefly characterized as follows [28,85]:

Mibefradil is a vasodilator agent, predominantly at arteriolar sites (resistance vessels) with significant negative chronotropic and negative dromotropic activity, whereas contractile force is not reduced by this agent, as shown in isolated organs, animal models, but also in patients [86,87]. The vasodilator activity of mibefradil is fully explained by its blocking effect on L-type calcium channels [87]. Mibefradil has a stronger effect on T-type than on L-type calcium channels, and it may well be that its negative chronotropic and dromotropic activities are brought about, at least in part, by T-type calcium channel blockade in the sinus node and in the A-V conduction system [28,85]. Interestingly, it was recently demonstrated in isolated human atrial tissue preparations that mibefradil in therapeutically relevant preparations also blocks N-type calcium channels. Consequently, noradrenaline release from cardiac sympathetic nerves is suppressed by mibefradil via N-type calcium channel blockade [88]. Similar findings have been obtained in isolated rat vessels [89]. Another interesting action of mibefradil was published recently by Mocanu et al. (1999) and by Schulz et al. (1999). In isolated rat hearts and in anesthetized pigs, mibefradil appeared to limit infarct size through a glibenclamide-sensitive mechanism, suggesting that mibefradil opens the ATP-dependent potassium channels [90,91].

Its clinical profile seemed promising, as it is an effective antihypertensive, displays no negative inotropic activity, protects against ventricular fibrillation in animal studies and also induces slight negative chronotropic activity. Moreover, it displays an inhibitory effect on hypertrophy of the heart and restenosis. However, despite this promising clinical profile, in June 1998 the Roche Company had to withdraw mibefradil from the market worldwide, because of its many interactions with other therapeutics used in cardiovascular medicine. The site of this interaction appeared to be the cytochrome P450 enzyme CYP3A4. Mibefradil interfered with the metabolism of these drugs, leading to increased plasma levels and thus to unwanted
side effects. The most noteworthy of the interactions between mibefradil and other drugs is that with the statin simvastatin [92].

Recently the MACH-1 study (Mortality assessment in Congestive Heart Failure has been published [93]. In this multicenter, randomized, double-blind study mibefradil was compared with placebo in 2590 patients with congestive heart failure (NYHA class II to IV) on top of their usual medication. Mortality and morbidity were assessed from 1996 to 1999. The interaction between mibefradil and anti-arrhythmic drugs, in particular amiodarone, and with the biotransformation of a number of other drugs might have caused the observed non-significant trend toward increased mortality, compared to placebo, in the first three months of the study. After this initial period this trend was not maintained: mibefradil did not show any advantage over placebo in this particular group of patients.

In spite of the aforementioned problems mibefradil must be considered as an interesting example of a CA which simultaneously blocks L-, T-, and N-type calcium channels. Accordingly, its value as a pharmacological tool is greatly appreciated.
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AIM OF THE PRESENT INVESTIGATION

Activation of the sympathetic nervous system is known to be associated with the treatment with calcium antagonists, in particular those of the dihydropyridine type. This sympathetic activation might be deleterious in patients with hypertension or angina pectoris, the major indications for the use of these drugs. It was a major aim of the present investigation to explore the differential interactions of various CA with certain components of the sympathetic nervous system. Because a rapid onset of vasodilator action by the CA activates the sympathetic nervous system via the baroreflex mechanism, the differential time courses of the vasodilator effects of various calcium antagonists were quantified in various rat models. Furthermore we investigated the inhibitory effect of mibefradil and verapamil on the N-type calcium channel (involved in the release of noradrenaline from the sympathetic nerve endings) as compared to the inhibitory effects of these effects on the L-type calcium channel.

Accordingly, the differential time courses of the vasodilator actions of calcium antagonists were quantified in rat and human isolated arteries, and also in situ in the forearm vascular bed of human volunteers, using venous occlusion plethysmography. A correlation with a physicochemical property of calcium antagonists, lipophilicity (denoted by the logarithm of the membrane-partition coefficient), was analyzed.

Electrical stimulation of both rat isolated tail arteries and the pithed rat preparation were used to evoke sympathetic noradrenaline release with subsequent contraction and increases in blood pressure and heart rate, respectively. The inhibitory effects of mibefradil and verapamil on these responses were quantified and differentiated from the inhibitory effects on the L-type calcium channel.