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Human vascular to cardiac tissue selectivity of L- and T-type calcium channel antagonists

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1 Voltage-operated calcium channel (VOCC) antagonists are effective antihypertensive and antianginal agents but they also depress myocardial contractility.

2 We compared four L-type calcium channel antagonists, felodipine, nifedipine, amlodipine and verapamil and a relatively T-type selective calcium channel antagonist, mibefradil, on human and rat isolated tissue assays to determine their functional vascular to cardiac tissue selectivity (V/C) ratio.

3 The V/C ratio was calculated as the ratio of the IC_{50} value of the antagonist that reduced (by 50%) submaximally contracted (K^+ 62 mM) human small arteries from the aortic vasa vasorum (vascular, V) mounted in a myograph and the IC_{50} value of the antagonist that reduced (−)-isoprenaline (6 nM) submaximally stimulated human right atrial trabeculae muscle (cardiac, C) mounted in organ chambers.

4 The average pIC_{50} values (−log IC_{50} M) for the human vascular preparations were felodipine 8.30, nifedipine 7.78, amlodipine 6.64, verapamil 6.26 and mibefradil 6.22. The average pIC_{50} values for the cardiac muscle were felodipine 7.21, nifedipine 6.95, verapamil 6.91, amlodipine 5.94, and mibefradil 4.61.

5 The V/C ratio calculated as antilog [pIC_{50}V-pIC_{50}C] is thus mibefradil 41, felodipine 12, nifedipine 7, amlodipine 5 and verapamil 0.2.

5 In rat small mesenteric arteries the pIC_{50} values for the five drugs were similar to the values for human vasa vasorum arteries contracted by K^+ 62 mM. However for methoxamine (10 μM) contraction in the rat arteries the pIC_{50} values were lower for felodipine 7.24 and nifedipine 6.23, but similar for verapamil 6.13, amlodipine 6.28 and mibefradil 5.91.

7 In conclusion, in the human tissue assays, the putative T-channel antagonist mibefradil shows the highest vascular to cardiac selectivity ratio; some 3 fold higher than the dihydropyridine, felodipine, and some 200 fold more vascular selective than the phenylalkylamine, verapamil. This favourable vascular to cardiac selectivity for mibefradil, from a new chemical class of VOCC antagonist, may be explained by its putative T-channel selectivity.

Keywords: Calcium antagonists; human trabeculae; human small arteries; amlodipine; felodipine; nifedipine; mibefradil; verapamil

Introduction

Drugs classified as specific L-type voltage-operated calcium channel (VOCC) antagonists (or calcium entry blocking agents) are important therapeutic agents for hypertension, angina pectoris, stroke, congestive heart failure, myocardial infarction and supraventricular tachycardia (Fleckenstein, 1983; Godfraind *et al*., 1986; Triggle, 1991). This class of drugs can be subclassified according to chemical structure into three main classes; the dihydropyridine class (i.e. nifedipine), phenylalkylamine class (i.e. verapamil) and benzothiazepine class (i.e. diltiazem). This subclassification also divides some cardiovascular properties where generally the dihydropyridines are more potent (effective at lower plasma concentrations) as vasodilating agents than depressing myocardial force (negative inotropy) or lowering heart rate and slowing atrioventricular conduction (Triggle, 1991). In contrast, verapamil and diltiazem do not display this relative vascular to cardiac selectivity so that in patients with congestive heart failure, peripheral dilatation and afterload reduction comes with a risk of further mycoardial depression. On the other hand, the vascular selective dihydropyridine drugs induce autonomic baroreceptor reflex tachycardia in response to the hypotension. Recently, a retrospective meta-analysis of short acting nifedipine preparations with a rapid onset of vasodilatation, has indicated a significant incidence of mortality or myocardial infarction perhaps associated with the reflex neurohumoral stimulation (see Pratt, 1997). Therefore, third generation calcium entry blocking agents have been sought with the following desirable properties: favourable pharmacokinetic properties; a vascular to cardiac selectivity; and a lack of neurohumoral stimulation.

A novel chemical structure, a tetralol derivative named mibefradil has been developed from some 500 derivatives of verapamil. In assay screens, it showed a selective coronary vasodilating property in Langendorff perfused guinea pig isolated heart at 12 fold lower concentrations than required to depress left ventricular pressure. This vascular selectivity was absent for verapamil (Clozel *et al*., 1991; Bühler *et al*., 1997). Subsequently, Mishra & Hermsmeyer (1994) showed that in voltage clamp studies in single cells from rat azygos vein, mibefradil had 30–100 times greater potency at halving the barium current for transient T-type calcium channels than for the long opening higher conductance L-type channels.

In contrast nisoldipine at a concentration that completely inhibited the L-channel current did not affect the T-channel current (Mishra & Hermsmeyer, 1994). Therefore for a fourth chemical class (tetralol) of VOCC antagonists, the novel property of T-over L-type calcium channel selectivity may explain the early results showing vascular selectivity in guinea pig isolated heart.

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In the present work, we sought to develop assays in human isolated tissue obtained at routine cardiac bypass operation to rigorously determine a functional quantitative measure of the vascular to cardiac muscle selectivity ratio of five calcium channel blocking drugs including three dihydropyridines, nifedipine, felodipine and amlodipine, a phenylakylamine, verapamil and the novel tetralol, mibefradil. We assessed the negative inotropic sensitivity in thin muscle strips from trabeculae from human right atrial appendage submaximally stimulated by (−)-isoprenaline. We chose relatively normal atrial muscle because of the more general availability and ease in setting up multiple thin strips. The vascular sensitivity was measured in a novel preparation of resistance arteries taken from the aortic vasa vasorum during routine coronary artery bypass graft surgery. The choice of this particular vessel was again governed by availability at cardiac surgery and reliability with which the small resistance arteries could be regularly dissected. Vessels were mounted in a myograph and precontracted by a K^+ depolarizing solution in the absence or presence of the calcium antagonist. In parallel studies, rat small mesenteric arteries were used to compare the relaxation potency of the calcium antagonists under either K^+ or methoxamine contraction stimuli. Neither the human atrial muscle nor the human vasa vasorum arteries may be the 'ideal' tissues to represent 'cardiac' and 'vascular' reactivity respectively. However we needed to compromise our desire for ideal tissues with what relatively non-diseased tissue could be ethically obtained from the operating theatre on a routine basis. The data show that mibefradil is the most vascular selective of the five drugs tested while verapamil was 5 fold more selective as a negative inotropic agent than a vasodilating agent. These selectivity ratios are the first to be determined in relatively normal human isolated tissue assays.

Methods

Preparation of human right atrial trabeculae

Human right atrial appendages were removed during routine coronary bypass graft surgery at the Royal Melbourne Public and Private Hospitals (Royal Melbourne Ethics approval 10/94, University of Melbourne, Human Research Ethics Committee No. 951686) and prepared as described previously (Gille *et al*., 1985; Molenaar *et al*., 1997). Atrial appendages were placed immediately into ice-cold pre-oxygenated modified Krebs' solution (mM): NaCl 98.5; KCl 5; $CaCl_2$ 2.25; $MgSO_4$ 0.5; $NaHCO_3$ 29; Na_2HPO_4 1; EDTA 0.04) and transferred from the operating theatre to the laboratory. Dissection of tissue containing intact trabeculae (<1 mm in diameter to facilitate oxygen and drug diffusion) under continuous oxygenation (5% CO_2 in O_2) commenced within 5–10 min of surgical removal. Each right atrial appendage yielded 1–11 muscle strips (average five) which were mounted in 50 ml tissue baths (Blinks, 1965) containing Krebs' solution at 37°C. Muscle strips were attached to strain-gauge transducers and driven with square wave pulses (1 Hz, 5 msec duration, just over threshold votage) *via* field electrodes. Two eight-bath racks were used. A length-tension curve was constructed to determine the length at which maximal contractions occurred (Lmax) and the tension adjusted to 50% Lmax to decrease reductions in basal tension over time (Gille *et al*., 1985). Tension of muscle strips were recorded on eight channel Watanabe recorders.

Responses to (−)-isoprenaline and calcium channel antagonists

The incubation medium was exchanged with Krebs' solution containing in addition (mM): disodium fumarate 5; sodium pyruvate 5; sodium glutamate 5 and ascorbic acid 2, to prevent catecholamine oxidation. When the tissues had stabilized, a cumulative concentration-effect curve was constructed to (−)-isoprenaline beginning at 0.2 nM using 1/2 log increments up to 0.2 μM. The tissues were washed and re-washed 30 min later with incubation solution as before. Following another 30 min, (−)-isoprenaline (6 nM), a concentration chosen to cause submaximal increase in contractile force, was added to the tissue bath. Upon equilibration, a single concentration of calcium antagonist: i.e. felodipine (10 nM–3 μM); nifedipine (3 nM–1 μM); verapamil (10 nM–10 μM); amlodipine (0.1 μM–0.1 mM); mibefradil (0.3 μM–0.1 mM) was added and the subsequent reduction in muscle contractility monitored for 2 h. Some tissues were designated as time controls and hence were not exposed to calcium antagonists. Note that only a single concentration of a calcium antagonist was tested on each trabeculae muscle. Tissue baths containing nifedipine, amlodipine and felodipine were protected from light by black polythene.

Human vasa vasorum small arteries

During routine coronary bypass graft surgery and preparation of the ascending aorta for end to side anastomosis of the saphenous vein graft, a 1–2 cm diameter patch of aortic vasa vasorum was dissected free. This tissue was obtained with ethical permission (see above). It was immediately placed in cool physiological saline solution (PSS) of the following composition (mM): NaCl 119; KCl 4.7; KH_2PO_4 1.18; $MgSO_4$ 1.17; $NaHCO_3$ 25; $CaCl_2$ 2.5; sodium edentate 0.026 and glucose 5.5 saturated with carbogen (5% CO_2 in O_2). The tissue was pinned out in a silastic (Sylgard®) based dish covered by cold PSS while one to four small arteries 2 mm long were dissected free with the aid of a dissecting microscope. Each vessel was mounted as a cylinder on 40 μM diameter stainless steel wires for isometric force recording in a dual channel myograph designed after Mulvany & Halpern (1997) (J.P. Trading, Denmark).

After warming the myograph to 37°C each vessel was stretched to an optimal circumferential length by moving one jaw *via* a micrometer. The increase in isometric force was made in steps to generate a length-tension relationship as previously described (Angus *et al*., 1988). From a computer fitted curve the vessel was finally stretched to L0.9 i.e. circumference of 90% of that where the vessel would be passively distended at 100 mmHg, determined from the Laplace relationship T = r.P (where P is transmural pressure of 100 mmHg, T = wall tension, r = internal radius). The diameter at this equivalent 100 mmHg (D100) was used to calibrate the internal diameter of the vessels. Force responses were recorded on a dual flatbed chart recorder (Model 320, W&W Scientific Instruments, Basel, Switzerland).

Protocol To stabilize the arteries, they were exposed to a depolarizing solution of K^+ 124 mM (KPSS) where Na^+ 124 mM was replaced by K^+ in PSS for 2 min. The chamber was replaced with PSS three times to rapidly restore the active contraction to KPSS back to baseline passive force.

Following this KPSS, the arteries were contracted with 50% KPSS (K^+ 62 mM with Na^+ 62 mM) as a routine depolarising and contracting stimulus every 30 min. Following this

exposure to 50% KPSS for 2 min and washing with three exchanges of PSS, a concentration of calcium antagonist or vehicle (DMSO) was added to the bath to equilibrate with the artery for 30 min prior to adding 50% KPSS with the same concentration of calcium antagonist. Again after 2 min, the bath was washed with PSS and a 0.5 log increment in calcium antagonist applied to the artery for a further 30 min equilibration. This was repeated five or six times to cover the concentration of antagonist needed to reduce the 50% KPSS response by >80%.

Rat mesenteric small arteries

Male Sprague Dawley rats aged between 6–7 weeks and weighing 200–300 g were anaesthetized with carbon dioxide (80%) in oxygen and killed by exanguination. A loop of intestine with mesentery attached was removed and placed in cooled PSS solution (see above). As for the vasa vasorum, a dissecting microscope was used to carefully dissect the second order branch of the mesenteric small artery free of fat and connective tissue. Two mm long segments were mounted as above and normalized in the myograph.

Protocol

As for the vasa vasorum small arteries, the mesenteric arteries were depolarized with K^+ KPSS (124 mM) for 2 min followed by PSS solution. This was repeated three times to ensure the stability and the viability of the preparations. To test the sensitivity of the calcium antagonists against both K^+ induced depolarization and receptor-operated contractions the following assays were conducted. The mesenteric arteries were exposed to the selective α_1-adrenoceptor agonist, methoxamine 10 μM (Figure 7). The contraction to methoxamine was repeated every 30 min after equilibration with increasing concentrations of calcium-entry blocking drugs or vehicle (DMSO). In a separate series of experiments, the five calcium entry blocking drugs were tested against K^+ 62 mM induced contractions in the same protocol as for the vasa vasorum.

Drugs used

The drugs used and their sources were: (−)-isoprenaline bitartrate (Sigma, St. Louis, MO, U.S.A.); nifedipine (Bayer, Basel, Switzerland); (±)-verapamil hydrochloride (Knoll AG, Australia); amlodipine besylate (Pfizer, Australia) and felodipine (Astra, Australia). Mibefradil (1**S**, 2**S**)-2-(2-[[3-(2-benzimidazolyl)propyl]methylamino]ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-napthyl-methoxyacetate dihydrochloride, has a molecular weight of 569, is a single enantiomer, chemically and light stable and water soluble (Roche, Australia). All other drugs used were of analytical grade. Stock solutions of (−)-isoprenaline (100 mM), verapamil (10 mM) and mibefradil (10 mM) were prepared in Milli-Q water. For the atrial muscle experiments stock solutions of nifedipine (10 mM), amlodipine (10 mM) and felodipine (10 mM) were prepared in ethanol. For the vessel assays, stock solutions of felodipine (0.1 mM) and nifedipine (1 mM) were prepared in dimethyl sulphoxide. All other drugs were dissolved and diluted in Milli-Q water. The dihydropyridines were protected from light during all experiments.

Data analysis

Atrial muscle The (−)-isoprenaline concentration-response curves were generated from the increase in isometric force above basal contraction to electrical field stimulation (Δ force) at each concentration of (−)-isoprenaline as a percentage of the maximum response to 0.2 μM (−)-isoprenaline (100%). The basal force plus the increase in force in response to (−)-isoprenaline 6 nM measured just prior to adding the calcium antagonist was taken as 100%. The total contraction height remaining at 30, 60, 90 and 120 min was measured as a percentage of the original height at time 0 (100%) from fast speed chart records.

Data are expressed as mean ± standard error of the mean (s.e.m.) for n muscles. The cumulative (−)-isoprenaline concentration-response curves were fitted with a logistic equation to estimate the pEC_{50}, ($-\log EC_{50}$) values. For the negative inotropic responses to the calcium antagonists, the concentration-response curve was constructed from a single response per tissue after 2 h exposure to the drug. The average fade in the contraction for the control tissues after 2 h was added to the percentage response in all drug treated tissues. Each concentration had 6–8 tissues, so a 3-parameter logistic equation:

$$E = a + \frac{b}{1 + e^{d(c + \log A)}}$$

where E is the response, a is starting level (100%), b is the range of response (100%), c is the pIC_{50}, d determines both slope and curvature and e is the base of the natural logarithm (Lew & Angus, 1995). The standard error of the pIC_{50} was estimated in the normal way from the matrix of covariance resulting from the iterative fitting process (Press *et al*, 1988).

Vasa vasorum and mesenteric small arteries The increase in contraction force above passive (stretch) force in response to K^+ 62 mM or methoxamine 10 μM was measured at the end of 2 min exposure and taken as 100% for the control or vehicle traces. Subsequent contractions at the end of 2 min exposures to K^+ 62 mM or methoxamine were calculated as a percentage of the control contraction. From each artery, a concentration–relaxation response curve was obtained. However some arteries could not be readily fitted with an individual logistic curve. Therefore on the computer screen, the lines through each point were joined and the IC_{50} concentration determined from the cursor values. These were averaged to give a pIC_{50} and the s.e.m calculated. Differences between means were tested by an unpaired Student's *t* test and $P<0.05$ considered to be significant. For multiple comparisons of pIC_{50} values for rat vessels contracted by K^+ and methoxamine, and human vessels contracted with K^+, one way ANOVAR with Tukey-Kramer multiple comparison *post-hoc* tests were used.

Vascular to cardiac selectivity ratio Vascular to cardiac selectivity ratio for each calcium antagonist was determined from the difference between the potency pIC_{50} value on the relaxation of human vasa vasorum arteries ($pIC_{50\ Artery}$) or the rat (K^+ 62 mM) or methoxamine (10 μM) precontracted arteries and the negative inotropic response in atrial trabeculae muscle ($pIC_{50\ Heart}$). The V/C ratio was calculated as a potency ratio: $V/C = \text{antilog}\ [pIC_{50\ Artery} - pIC_{50\ Heart}]$.

Results

Patient history and drug therapy

In contrast to experimental animal tissue, human isolated tissue is subject to a wide range of variability from age, disease status and preisolation drug therapy. The majority of atrial

appendages in the present study were from patients undergoing coronary artery bypass graft surgery (>90%), were male (>70%) and with an average age of 65 (Table 1). Their prior drug treatment was generally a β-adrenoceptor antagonist, a calcium antagonist and nitrates. The tissues previously exposed to a calcium channel antagonist (never mibefradil) were evenly spread among the subsequent five calcium antagonists or time control group with >46% of all tissues being taken from patients on calcium antagonists at the time of surgery.

Because of the choice to submaximally stimulate the tissues with (−)-isoprenaline, it was important to know whether therapy of β-adrenoceptor antagonists prior to surgery affected the organ chamber sensitivity to (−)-isoprenaline. The (−)-isoprenaline concentration-response curves in tisssues from 28 patients exposed to metoprolol, atenolol or sotolol were pooled and compared with tissues ($n=31$) without prior β-adrenoceptor antagonist treatment. The subsequent concentration response curves to (−)-isoprenaline were not different in sensitivity with β-adrenoceptor antagonist treatment (pEC_{50} 8.56 ± 0.02) and without prior treatment (pEC_{50} 8.37 ± 0.03), $P=0.09$, unpaired Student's t test.

Therefore we have not selected any of the atrial muscle for special consideration on the grounds of prior drug treatment. Given that the vasa vasorum was also from coronary artery bypass graft patients we assume that similar presurgery drug therapy was used.

Atrial trabeculae contractions

The basal force to field stimulation fell after the concentration-response curve to (−)-isoprenaline in each muscle. We chose to use a concentration of 6 nM (−)-isoprenaline, just above the EC_{50} values of 3 and 4 nM in the previous concentration-response assays (tissues with and without β-adrenoceptor antagonist exposure) to submaximally contract the muscle to a steady level prior to the addition of the calcium antagonist. Prior to the subsequent submaximal stimulation with 6 nM (−)-isoprenaline the basal force ranged from 1.98 ± 0.3 mN for the verapamil cohort to 5.16 ± 0.72 for the felodipine group. The subsequent inotropic response (Δ force above basal) to (−)-isoprenaline 6 nM ranged from 4.96 ± 0.57 to 7.74 ± 1.56 mN. Importantly the basal contractile force contributed between 36–40% of the starting total force (100%) after (−)-isoprenaline in the five groups just before the exposure to the calcium antagonist.

Negative inotropic action of calcium antagonists

The time dependent fade of the total force (field and (−)-isoprenaline response) was $23.9\pm3.5\%$, ($n=11$) (Figures 1 and 2). From pilot experiments it was clear that the fall in inotropic response to lower concentrations of calcium antagonists, especially felodipine, needed greater than 60 min to come close to equilibrium. We therefore defined a 120 min exposure time for all drugs. The families of time dependent falls in contractile force over 2 h are shown for two of the five calcium antagonists (with the control group (absence of antagonist) repeated on each graph for felodipine and mibefradil, Figures 2 and 3). From the average data at 2 h corrected for the fade in the untreated tissue, the family of data points for each drug were fitted with the logistic (Figure 4). The fitted pIC_{50} values show that in this assay the least potent negative inotropic calcium antagonist was mibefradil with a pIC_{50} 4.61 ± 0.18 (Table 2). If this is given a potency of 1 then relative to mibefradil, the rank order of potency is felodipine (398) > nifedipine (218) > verapamil (200) > amlodipine (21) > mibefradil (1).

Human vasa vasorum arteries

After the length-tension normalization protocol, the small arteries ($n=5-6$) grouped according to subsequent treatment were estimated to have internal diameters at equivalent transmural pressure of 100 mmHg (D100) of: felodipine 365 ± 51 μm; amlodipine 304 ± 12 μm; verapamil 301 ± 12 μm; mibefradil 330 ± 17 μm; nifedipine 253 ± 31 μm and vehicle (DMSO) 359 ± 31 μm. The DMSO vehicle treated tissues showed a small increase in contraction to K^+ 62 mM over five contractions of $9.2\pm11.4\%$ (Figure 5). This has not been factored into subsequent responses to the calcium antagonists.

Table 1 Summary of patient histories, surgical procedure and properative therapy for each group of atrial appendages as subsequently treated with calcium antagonist

Drug tested	*Nifedipine*	*Verapamil*	*Felodipine*	*Amlodipine*	*Mibefradil*	*Nil**
n (*patients*)	*18*	*18*	*13*	*18*	*13*	*11*
Sex	4F/14M	5F/13M	2F/11M	4F/14M	4F/9M	2F/9M
Age (mean ± s.e.m.)	63 ± 2	68 ± 2	67 ± 3	64 ± 2	63 ± 4	65 ± 3
Surgical procedure						
AVR*	0	0	0	1	1	0
CABG*	16	17	12	16	10	10
MVR*	0	0	0	0	1	0
AVR/CABG	2	0	1	0	1	1
MVR/CABG	0	1	0	1	0	0
Drugs						
β-adrenoceptor antagonists	11	11	3	11	5	9
Ca^{2+} antagonists	10	10	6	7	8	5
ACE inhibitors	5	5	4	3	3	3
Diuretics	3	2	1	5	3	2
Hypolipidemics	3	2	6	2	0	1
Nitrates	11	11	7	9	5	7
Hypoglycemics	3	5	2	1	1	1

*AVR = aortic valve replacement; *CABG = coronary artery bypass graft; *MVR = mitral valve replacement; *Nil = tissues used to determine time dependent fade in contraction over 2 h.

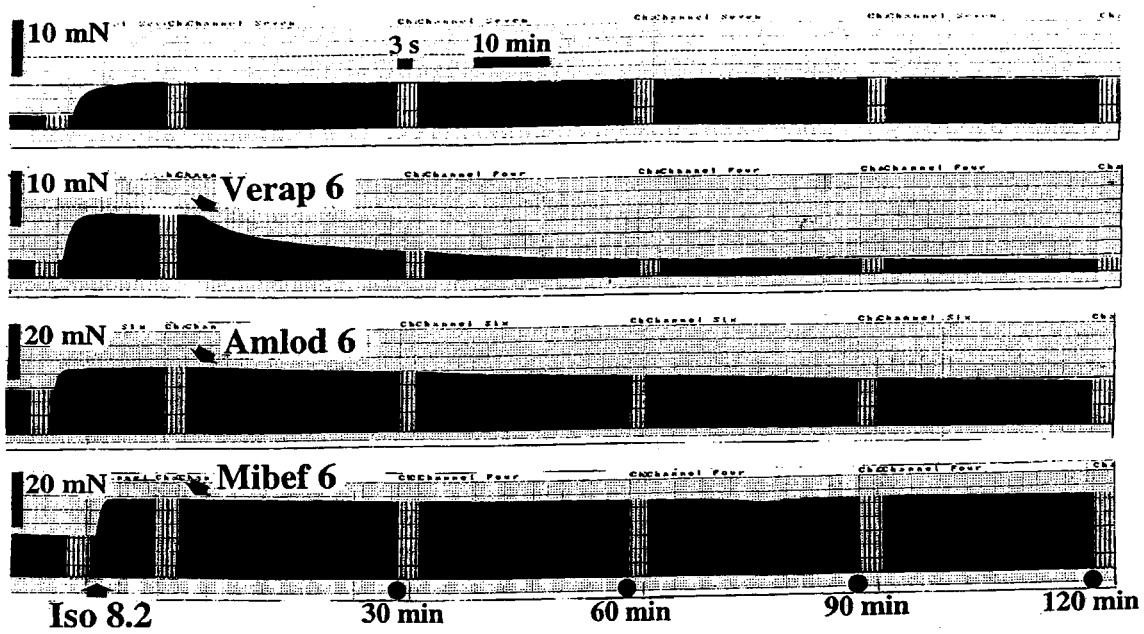

Figure 1 Experimental protocol used for determining potency of calcium antagonists in human atrial trabeculae. Shown are original recordings from four patients who presented for coronary grafting surgery. The top trace shows a time control recording for 120 min from a 72 year-old female following stimulation by a single concentration of (−)-isoprenaline (Iso) shown as −log [agonist], M (i.e. 8.2 = 6 nM). The next three recordings show the effects of calcium antagonists on the positive inotropic effect elicited by Iso. The second trace shows the effects of verapamil (Verap) from a 68 year-old male; the third trace, the effects of amlodipine (Amlod) for a 47 year-old male and the bottom trace, the effects of mibefradil (Mibef) from a 72 year-old male all at a −-log [antagonist], M, of 6 (i.e. 1 μM) over 120 min.

The estimates of pIC_{50} for the five calcium antagonists show that the most potent vasorelaxant drug in this assay was felodipine pIC_{50} 8.30 ± 0.19, followed by nifedipine (3.3 fold less potent), amlodipine (46), verapamil (109) and mibefradil (120), (Table 2, Figure 6). We also attempted to test the calcium antagonists at inhibiting vasa vasorum arteries contracted by receptor-mediated stimuli rather than simply K^+ induced depolarization. However, noradrenaline, methoxamine, angiotensin II, and vasopressin were very poor or inactive constrictor agents of the vasa vasorum arteries compared with K^+ 62 mM. The only sustained contraction achieved was in response to endothelin 1 but repetitive contractions could not be obtained and this approach was abandoned. Therefore, given the paucity of human vasa vasorum arteries, we could not develop a viable assay with endothelin 1 as the contraction stimulus.

Rat mesenteric arteries

The average D100 internal diameters of the rat mesenteric arteries for the subsequent contractions by K^+ 62 mM were felodipine 433 ± 18 μm, amlodipine 445 ± 15 μm, verapamil 355 ± 11 μm, mibifradil 396 ± 16 μm, nifedipine 397 ± 28 μm and vehicle (DMSO) 410 ± 18 μm. Over time, the DMSO treated tissues showed a small increase in force of 9.18 ± 11.4% (Figure 6). The potencies for the five calcium antagonists at inhibiting the K^+ 62 mM contracted rat mesenteric arteries were not significantly different to the potencies (pIC_{50}) displayed in the K^+ 62 mM contracted human vasa vasorum arteries (Table 2). For example, the human and rat artery pIC_{50} values for the most potent calcium antagonist felodipine were 8.30 ± 0.19 and 8.42 ± 0.13 (NS) and for the least potent drug, mibefradil, were 6.22 ± 0.11 and 6.50 ± 0.11 (NS) respectively (Table 2).

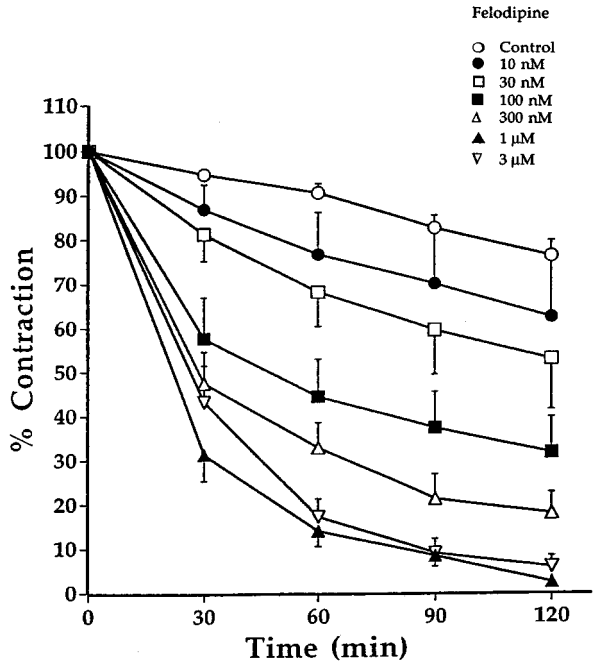

Figure 2 Time course of inhibition of the positive inotropic response of (−)-isoprenaline 6 nM in the absence or presence of felodipine (10 nM − 3 μM) on human right atrial trabeculae. Each point represents the mean values from 6 − 8 patients and the bars are ± 1 s.e.m. Results are given as per cent of equilibrium response (100%) to (−)-isoprenaline 6 nM. Only one concentration of calcium antagonist was applied to each tissue.

For the rat arteries exposed to methoxamine (10 μM), the D100 diameters in the different groups were, felodipine 392 ± 26 μm, amlodipine 416 ± 11 μm, verapamil 407 ± 21 μm,

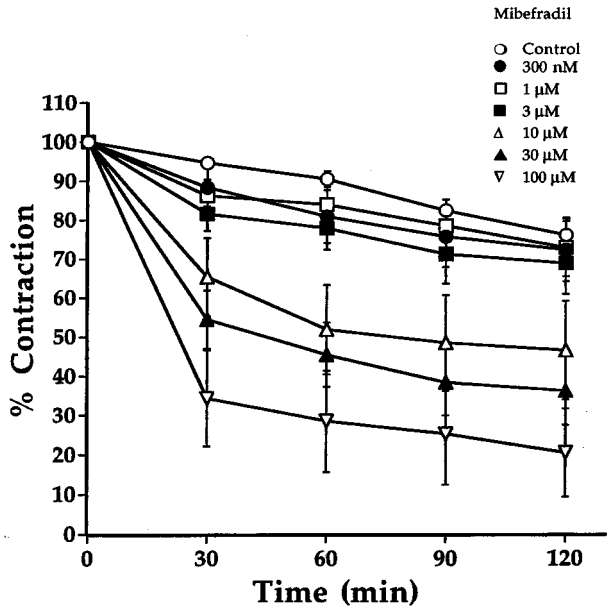

Figure 3 Time course of inhibition of the positive inotropic response of (−)-isoprenaline 6 nM in the absence or presence of mibefradil (300 nM–100 μM) on human right atrial trabeculae. Each point represents the mean values from six to eight patients and the bars are $\pm$1 s.e.m. Results are given as per cent of equilibrium response (100%) to (−)-isoprenaline 6 nM. Only one concentration of calcium antagonist was applied to each tissue.

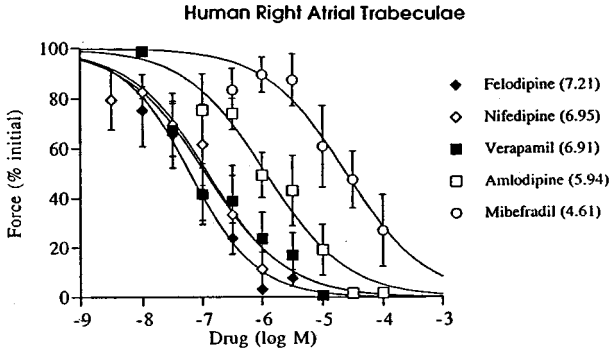

Figure 4 Average negative inotropic responses to five calcium channel antagonists in human isolated right atrial trabeculae. Each average value and $\pm$1 s.e.m. bar is the inotropic force at 2 h for six to eight tissues in the presence of a concentration of calcium antagonist. This force is calculated as the % of the initial response to a submaximal concentrtion of (−)-isoprenaline (6 nM) taken as 100%. Note, only one concentration of calcium antagonist was applied per tissue. The values in brackets are the pIC_{50} values ($-\log IC_{50}$) calculated by fitting a logistic equation (see Methods).

mibefradil 389$\pm$31 μm and vehicle 365$\pm$27 μm (H_2O) time control. Just as for the K^+ 62 mM precontracted arteries, the 2 min contraction to methoxamine (10 μM) increased over time by 26.04$\pm$5.9% compared to the first contraction to methoxamine when no calcium antagonist was used (Figure 7).

Unlike the K^+ 62 mM contraction in the human or rat arteries, the α_1-adrenoceptor mediated contraction by methoxamine was inhibited by the dihydropyridines, felodipine and nifedipine in a manner suggesting two components. The lower concentrations of the calcium antagonist (0.01–0.1 μM) caused some 40–50% inhibition of methoxamine contractions but concentrations greater than 1 μM were required to complete the inhibition (Figures 7 and 8). Comparing nifedipine pIC_{50} values across the three vascular assays, it was 1.57 log units, i.e. 37 fold less potent in methoxamine contracted rat arteries than K^+ contracted arteries ($P<0.05$) and 1.65 log units (45 fold) less potent comparing rat (methoxamine) to the human vasa vasorum (K^+) assay. Within the rat mesenteric artery assay, the calcium blocking drugs were generally less potent in the

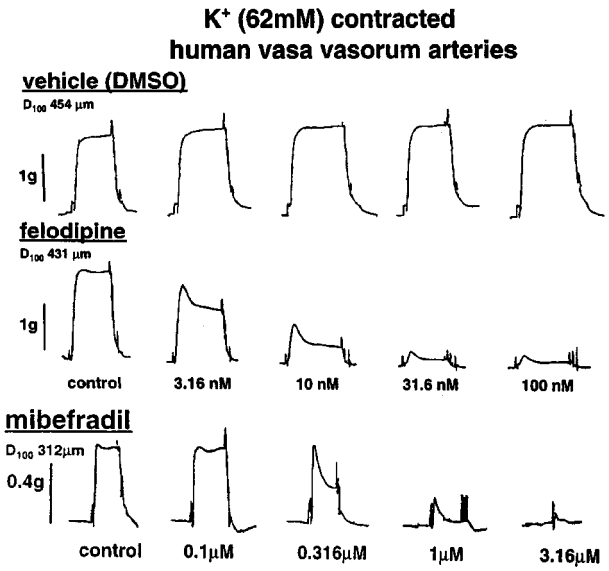

Figure 5 Chart records of isometric contraction of three human isolated small arteries from aortic vasa vasorum of 454 μm, 431 μm and 312 μm internal diameter in response to 2 min exposures to KCl (K^+ 62 mM) repeated every 30 min. Vehicle trace (top): concentrations of DMSO (left to right) were 0, 0.03%, 0.1%, 0.3% and 0.1% Middle trace: felodipine; 0–100 nM in 0, 0.03, 0.1, 0.3 and 0.1% DMSO. Bottom trace: mibefradil 0–3.16 μM in H_2O. DMSO vehicle, felodipine and mibefradil were added 30 min prior to retesting each K^+ 62 mM exposure. The sharp vertical marks are associated with changing the bath solution.

Table 2 Average potency pIC_{50} of five calcium antagonists inhibiting the contraction of rat small mesenteric arteries contracted by methoxamine 10 μM (Rat Meox) or by K^+ 62 mM (Rat K^+) or human isolated vasa vasorum small arteries contracted by K^+ 62 mM (Human K^+) compared with human atrial trabeculae muscle contracted by isoprenaline (atrial muscle)

	Small artery							
	Rat (Meox)		*Rat (K^+)*		*Human (K^+)*		*Atrial muscle*	
Mibefradil	5.91$\pm$0.1†	(6)	6.50$\pm$0.11	(5)	6.22$\pm$0.11	(5)	4.61$\pm$0.18	(36)
Felodipine	7.24$\pm$0.42	(6)	8.42$\pm$0.13	(4)	8.30$\pm$0.19	(6)	7.21$\pm$0.12	(45)
Nifedipine	6.23$\pm$0.32†*	(6)	7.80$\pm$0.14	(5)	7.78$\pm$0.13	(6)	6.95$\pm$0.17	(36)
Amlodipine	6.28$\pm$0.10*	(5)	6.59$\pm$0.08	(5)	6.64$\pm$0.07	(4)	5.94$\pm$0.14	(42)
Verapamil	6.13$\pm$0.08	(5)	6.36$\pm$0.06	(5)	6.26$\pm$0.13	(5)	6.91$\pm$0.14	(42)

Values are mean$\pm$1 s.e.m. from n tissues (*n*); †ANOVAR, Tukey-Kramer *post-hoc* test: rat (Meox) versus K^+ $P<0.05$; *Rat (Meox) versus human K^+ $P<0.05$.

methoxamine assay compared with K^+ assay. The differences in potency were nifedipine 37 fold, felodipine 15 fold, mibefradil 3.9 fold, amlodipine 2.0 fold and verapamil 1.7 fold.

Vascular to cardiac selectivity ratio

Table 3 shows the human artery (vasa vasorum) and heart (atrial trabeculae muscle) pIC_{50} values and their difference (log Δ) for each calcium antagonist. The antilog gives the V/C potency ratio indicating that mibrefradil has the greatest potency ratio being 40 fold more selective at relaxing the vasa vasorum artery than depressing the atrial trabeculae muscle. Of interest is the ratio of 0.22 for verapamil indicating a 5 fold cardiac selectivity over vascular relaxation. Thus, compared with verapamil (if given a ratio of 1) the vascular to cardiac

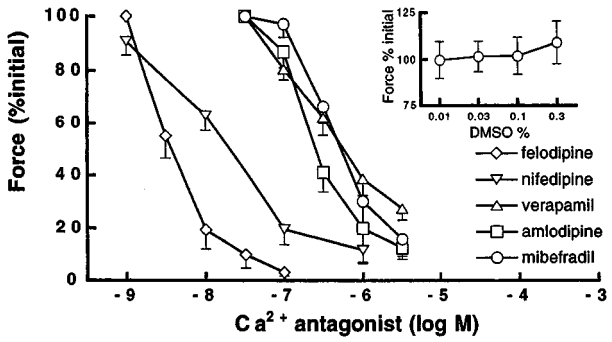

Figure 6 Average contraction responses of human isolated arteries from aortic vasa vasorum exposed to K^+ (62 mM) in the presence of increasing concentration of five calcium antagonists. Symbols are average responses (± 1 s.e.m. error bars) at specific concentrations generated from cumulative curves within artery. Responses were calculated as % of initial 2 min exposure to K^+ 62 mM. Inset shows average effects of DMSO cumulative concentrations on the contraction to K^+ 62 mM ($n = 5$ arteries).

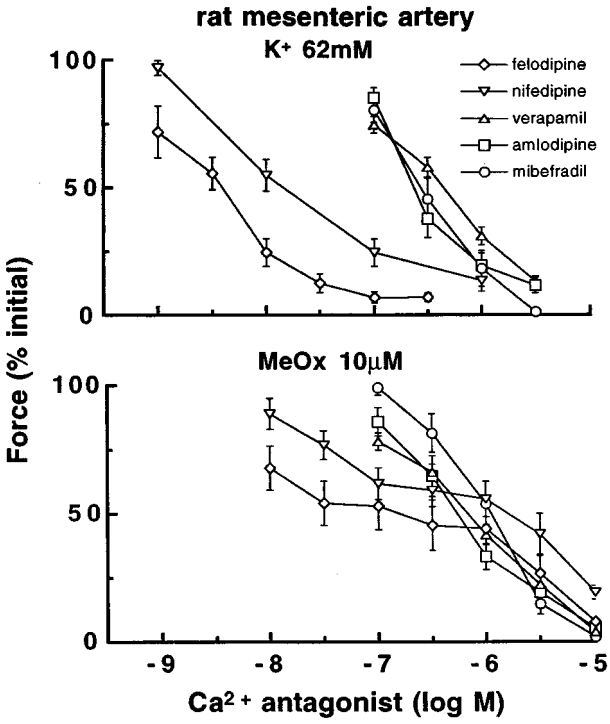

Figure 8 Average contraction responses of rat isolated mesenteric small arteries exposed for 2 min to K^+ (62 mM), top; and methoxamine (10 μM), bottom; in the presence of increasing concentrations (30 min exposure) of five calcium antagonists. Symbols are average responses (± 1 s.e.m. error bars) at specific concentrations generated from cumulative curves within artery. Responses were calculated as % of initial 2 min exposure to K^+ (62 mM) or methoxamine 10 μM.

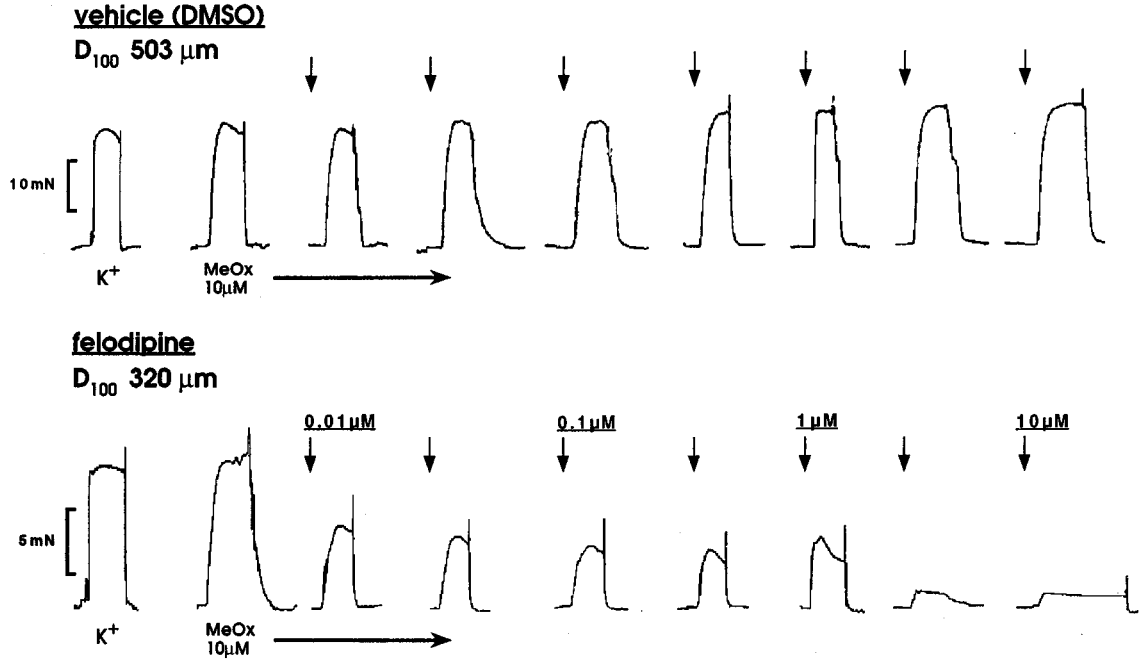

Figure 7 Chart records of isometric contractions of two rat isolated mesentric small arteries of 503 μm and 320 μm internal diameter in response to 2 min exposures to methoxamine (10 μM) repeated every 30 min. Vehicle trace (top) DMSO concentrations (%) at first arrow (left to right) are 0.01, 0.03, 0.1, 0.03, 0.1, 0.3 and 0.1%. Bottom trace: felodipine added to bath at arrow (left to right) 0.01 – 10 μM in 0.5 log units 30 min prior to subsequent exposure to methoxamine (Meox). The sharp vertical spikes at end of 2 min exposure to Meox are associated with changing the bath solution.

selectivity of the remaining four drugs is mibefradil 185; felodipine 56; nifedipine 31 and amlodipine 23.

If the rat mesenteric artery pIC_{50} data with K^+ contraction is used with the human atrial muscle data, the V/C potency ratios are very similar to the human (K^+) : human muscle ratios (Table 3). But if the potency of calcium antagonists to relax methoxamine contracted arteries of rat mesentery were compared with the potency to inhibit isoprenaline induced inotropic responses in human atrial muscle, the ratios of Vascular to Cardiac selectivity are: (antilog[pIC_{50} rat Meox – pIC_{50} human isoprenaline) felodipine 1.07; nifedipine 0.2; amlodipine 2.2; verapamil 0.16; and mibefradil 20. The only vascular to cardiac selective calcium antagonist under these conditions was mibefradil. In contrast, both nifedipine and verapamil were 5 or more fold more potent as cardiac depressants compared with their potency as vasodilator agents while felodipine and amlodipine were barely vascular selective.

Discussion

Our findings show that five drugs classified as voltage-operated calcium channel (VOCC) blocking drugs display very different potencies as negative inotropic agents and relaxants of vascular tissue. The power of the present work comes from the comparative value of five agents tested in the same laboratory on highly refined assays on human isolated tissue and on rat mesenteric small arteries. The relevance of the findings to therapeutics is not the absolute pIC_{50} for each drug on each assay but the comparative ratio of potency between artery and heart muscle among the five drugs that will allow some prediction for their different cardiovascular behaviour in man.

Atrial preparations

Atrial trabeculae muscle strips provide a convenient source of myocardium that is not likely to be grossly diseased or failing in the patient cohort we used. Other studies have used left ventricular papillary muscle preparations from explanted hearts removed because of heart failure (Cremers *et al*., 1997). However, atrial muscle may have different VOCC characteristics from ventricular muscle.

Our patients have received a wide range of drugs prior to surgery. While this is inevitable, we do not believe this should grossly distort the potency of the calcium antagonists in the isolated tissue assay since (i) the tissues with or without prior exposure to β-adrenoceptor antagonists did not have significantly different sensitivities to (−)-isoprenaline, and (ii) our estimate of the pIC_{50} for nifedipine in vasa vasorum under K^+ contraction was 7.78 ± 0.13 which is not significantly different to 7.71 ± 0.23 reported in dog saphenous vein or 7.50 ± 0.12 in dog large coronary artery where no prior drug therapy was used (He *et al*., 1988).

We chose to drive the atrial muscle strips with field electrode stimulation just above a threshold voltage to avoid depolarizing sympathetic nerves. This electrical stimulus would depolarize the sarcolemma and open the L-type VOCC. To enhance this calcium entry, the tissues were submaximally stimulated by (−)-isoprenaline which activates β_1- and β_2-adrenoceptors causing accumulation of cyclic AMP, activation of cyclic AMP dependent protein kinase and phosphorylation of the L-type VOCC (Kaumann & Molenaar, 1997). As the data show, both the resting (basal) force and (−)-isoprenaline stimulated inotropic force are inhibited by all five drugs at high concentration. Various protocols have been used to assess the potency of calcium antagonists. In pilot experiments we noted the long (>30 min) time needed to allow the decrease in force to reach a plateau especially with low concentrations and the complication with time-dependent fade in the contractile force in vehicle-treated muscle (Kjellstedt *et al*., 1985; Ljung *et al*., 1987). Slow onset may be a feature of some VOCC antagonists such as exhibited by amlodipine (Burges *et al*., 1985; Arrowsmith *et al*., 1986; Matlib *et al*., 1988; Stopher *et al*., 1988). Thus rather than use intervals of 60 min between dosing in a cumulative concentration-response protocol (Godfraind *et al*., 1984) or worse 30 min (Magnon *et al*., 1995) we chose to measure the effect of just a single concentration in each tissue after 120 min equilibration and adjust the responses for the fade in contractile force observed in non-treated muscle. Data analysis in this design is less straightforward because single concentrations were used per tissue (Angus *et al*., 1980). Thus, rather than individual tissue IC_{50} concentrations being generated per tissue, a global estimate was found by an iterative fitting process for the entire data set for that drug.

Table 3 Vascular to cardiac potency (selectivity) ratios for calcium antagonists calculated from Table 2

Vascular preparation:	*Human (K^+)*	*Rat (K^+)*	*Rat (Meox)*
Cardiac tissue:	*Human*	*Human*	*Human*
Mibefradil	40.7	77.6	20
Felopidine	12.3	16.2	1.1
Nifedipine	6.8	7.1	0.2
Amlodipine	5.0	4.5	2.2
Verapamil	0.22	0.28	0.16

The selectivity ratios are calculated from mean pIC_{50} values from Table 2 as antilog [pIC_{50} vascular–pIC_{50} cardiac]. The three ratios for each calcium antagonist relate to the three pIC_{50} for the vascular preparation (Table 2); human vasa vasorum arteries contracted by K^+ 62 mM (K^+), rat mesenteric small arteries contracted by K^+ 62 mM (K^+) or methoxamine (Meox) and the cardiac pIC_{50} potency from human atrial muscle used in all three ratio calculations. (Ratios less than 1 are cardiac selective).

Human vasa vasorum

Ideally, human large and small coronary arteries would be valuable tissues in which to seek a vascular relaxation potency for the five drugs for comparison with myocardium. Tissue selection is a key point as estimates of pIC_{50} will, in part, be dependent on the location of ion channels, the preconstrictor agent, the thickness of the artery wall and equilibration time. The ascending aortic vasa vasorum is a vascular network with origins from branches of the right coronary artery running in the adventitial layer that supplies nutrition for the aortic wall (Stefandis *et al*., 1995). The vessels are small diameter resistance arteries that contract to a maximum in less than 1 min, in response to a rise in extracellular K^+ concentration, because of the rapid diffusion across the relatively thin wall. The depolarization induced by elevation of extracellular K^+ is mainly due to the variation of the equilibrium potential of potassium (E_K).

In testing VOCC antagonists, many studies have shown that the L-type VOCC antagonists are most potent in relaxing arteries precontracted by K^+ depolarization compared with receptor-stimulated constrictor agents such as endothelin, serotonin or thromboxane mimetics (Angus & Brazenor, 1983) in human large internal mammary artery (He *et al*., 1989) and saphenous vein (He *et al*., 1993). K^+ depolarizing solution may also depolarize sympathetic nerves and release noradrenaline. However in these vasa vasorum arteries the

response to electrical field stimulation is negligible and there is essentially no contraction to methoxamine suggesting little role for sympathetic innervation or postjunctional α_1-adrenoceptors (Angus & Fujiwara, unpublished observation). While a rise in extracellular K^+ is highly unlikely to be a natural stimulus *in vivo*, it was chosen to precontract the arteries because of its clear mechanism at indirectly opening voltage-operated calcium channels.

Given that K^+ mediated contraction could bias the results in favour of one of the calcium antagonists that selectively blocked voltage-operated calcium channels at the low-voltage end (i.e. Mibefradil at 'T' channels), we tried testing the range of calcium antagonists against a receptor-operated constrictor stimulus. We found that the receptor operated agonists vasopressin, angiotensin II, methoxamine and noradrenaline did not cause significant contractions of the vasa vasorum arteries. The only significant contraction was to endothelin 1 but the lack of repetitive contraction due to a very slow washout precluded further experiments. Therefore we were unable to define a potency in the vasa vasorum for the calcium antagonists for inhibiting receptor-mediated contractions.

Therefore to answer the question of potential constrictor bias in the vascular selectivity, we chose the rat mesenteric artery preparation that gave reproducible contractions to K^+ 62 mM and to methoxamine, the selective α_1-adrenoceptor agonist that is not subject to neuronal uptake and is readily washed from the tissue.

It was important to establish whether, under K^+ depolarization, the rat mesenteric artery relaxed to the calcium antagonists with similar pIC_{50} values as in the human vasa vasorum arteries. We found that there was no significant difference in relaxation potency between the human and rat small arteries contracted by K^+ for any calcium antagonist (Table 2). Thus it could be reasonably assumed that for the receptor-operated contraction by methoxamine the rat mesenteric artery could offer some guide for the potency of calcium antagonists in human small arteries were such receptors to be found.

In the rat mesenteric artery we were surprised to find that under methoxamine contraction, there appeared to be two components to the concentration-relaxation curves for nifedipine and felodipine; a potent early relaxation of approximately 50% and a weak high concentration component. In contrast verapamil, amlodipine and mibefradil concentration-relaxation curves were apparently normal but the three drugs were less potent compared with pIC_{50} estimates for K^+ contractions.

Human vascular to cardiac selectivity

The strength of the present assays of human vascular and cardiac activity of this group of VOCC antagonists is that they were internally equivalent. Amongst the VOCC drugs tested, the major outlier was verapamil. In our assays verapamil displayed a cardiac to vascular selectivity of 5 fold while the remaining four drugs were vascular to cardiac selective. The results show that nifedipine and amlodipine were equivalent in their vascular to cardiac selectivity of approximately 5 fold while felodipine was 12 fold and mibefradil 40 fold. If these selectivities are compared with verapamil, then the V/C ratios should be multiplied by 5 giving mibefradil a 200 fold more vascular to cardiac selectivity compared with verapamil.

An important question is how much vascular to cardiac selectivity is necessary before a therapeutic difference is exhibited. One measure is to examine the plasma levels of the drug. Cremers *et al.* (1997) suggested that the therapeutic active plasma concentration (TAPC) was 0.14 μM for verapamil and nifedipine, and 0.87 μM for mibefradil (see Henry, 1980; McAllister & Kirsten, 1982; Clozel *et al.*, 1991). If these plasma concentrations are in equilibrium with the vascular and cardiac calcium channels then our studies suggest that verapamil would give 20–30% relaxation of small arteries but more than 70% inhibition of myocardial force, indicating a preferential effect on myocardium. Nifedipine at -6.85 log M would relax an artery to 20% and depress the myocardium to 40%, while mibefradil at -6.06 log M would relax the artery to 30% and have very little effect on the myocardium suggesting a substantial vascular selectivity.

Published reports from *in vitro* studies show similar vascular to cardiac selectivity ratios for verapamil (1) and nifedipine (14) from rat portal vein and papillary muscle (Ljung, 1985) as found here. *In vivo* studies of hypotension and bradycardia in anaesthetized rats with autonomic reflex blockade have been used recently to reflect vascular to cardiac selectivity of dihydropyridines (Nordlander *et al.*, 1995). Amlodipine, felodipine and nifedipine had vascular/cardiac selectivity ratios of 15, 121 and 47 respectively in these *in vivo* assays. However in assays of isolated perfused rat hearts, the sensitivity of fall in heart rate and dP/dt were similar for amlodipine and felodipine, but nifedipine was 20 fold force/rate selective (Nordlander *et al.*, 1995). Therefore, it would seem prudent not to extrapolate from negative inotropic sensitivity to include chronotropic sensitivity at least for the dihydropyridines.

The explanation for mibefradil having a significant vascular selectivity over depressing myocardial contractility may be related to the apparent T to L-type VOCC selectivity of this drug. At present there are no electrophysiological studies of voltage clamp in isolated cells from human atrial muscle or vasa vasorum. Whether there are T-channels in human atrial muscle is uncertain. If they are sparse in number, or play little role in terms of calcium entry for basal and stimulated contractility, mibefradil would be expected to have no negative inotropic activity. Unlike other reports in human papillary muscle (Cremers *et al.*, 1997) we have demonstrated that given sufficient concentration, mibefradil will inhibit myocardial force perhaps as the drug inhibits calcium entry through L-channels. This would be consistent with mibefradil having only a relative T- to L-channel selectivity. To be consistent with this argument, we would conclude that human vasa vasorum and rat mesenteric arteries have important functional T-channels activated by raised K^+ and membrane depolarization or methoxamine and similar to those found in rat vascular muscle cells (Mishra & Hermsmeyer, 1994). In voltage clamp studies in single rat vascular smooth muscle cells, mibefradil was 100 times more potent at inhibiting the barium current (I_{Ba}) on T-type calcium channels (IC_{50} 0.1 μM) compared with L-type calcium channel (IC_{50} 10 μM) (Mishra & Hermsmeyer, 1994). The experimental conditions under which the heart and vascular muscle were tested in the present work may also have led to the apparent vascular selectivity for four of the five drugs. In adult normal atria the maximum diastolic membrane potential was -79.4 ± 1 mV. In the same study the action potential amplitude was 63.2 mV in the absence, and 81.7 mV in the presence of maximum β-adrenoceptor stimulation with adrenaline (Mary-Rabine *et al.*, 1980). The atrial muscle would be depolarized/repolarized continuously at 1 Hz while the blood vessel would be at its resting Em for 30 min in the presence of the calcium antagonist before the 2 min exposure to K^+ 62 mM and depolarization. In rat mesenteric small arteries (326 μm diameter) K^+ 60 mM depolarized the arteries mounted in a similar myograph as used in the present study,

from − 60 to −25 mV (Fujiwara & Angus, 1996). In a detailed study in 200 μm rat mesenteric resistance arteries, noradrenaline 10 μM depolarized the membrane from -59 ± 0.4 to -33.7 ± 1.0 mV while K^+ 125 mM caused the membrane to depolarize to -6.9 ± 1.7 mV although the contraction was only 70% of that to noradrenaline (Mulvany *et al*., 1982). Whether these conditions affect the measured potency is unknown. Again the comparative data for the five drugs tested under the same conditions allows some comfort for drawing inferences.

If the vascular to cardiac selectivity ratio in these human tissue assays is asserted to be of little relevance because of K^+ induced depolarization, the data from the methoxamine contracted rat mesenteric artery ought to be of some importance. Mibefradil still retains 20 fold vascular to cardiac selectivity markedly different from felodipine (1.1) and amlodipine (2.2). Of interest is the reverse selectivity for nifedipine (0.2) and verapamil (0.16) showing cardiac depression over vascular relaxation selectivity. Thus whether the vascular preparation is depolarized by K^+ or contracted by α-adrenoceptor mediated contraction, the 'T' channel selective antagonist, mibefradil, appears to be vascular selective and stand apart from the other four VOCC drugs tested.

Recently, at least for the dihydropyridine VOCC, there is evidence that variation in the voltage-gated channel protein expressed by the α_{IC} gene leads to vascular to cardiac selectivity. Welling *et al*. (1997) showed that spliced variants of the IS6 segment of the α_{IC} gene ($\alpha_{IC\text{-}a}$ and $\alpha_{IC\text{-}b}$) were specifically located in rat ventricular myocytes ($\alpha_{IC\text{-}a}$) and in rat aorta ($\alpha_{IC\text{-}b}$) and that nisoldipine showed greater sensitivity against the $\alpha_{IC\text{-}b}$ vascular L-type channel than against the $\alpha_{IC\text{-}a}$ cardiac channel. Thus the basis for vascular to cardiac selectivity of VOCC antagonists may involve the density and location of the broad type of VOCC (T and L), the possibility of alternative spliced segments of the critical voltage-gated pore protein, if located to specific tissues, and the resting membrane potential. The 'T' channel is rat brain has been cloned and the authors report that they have identified human and mouse homologues. This T-type channel has the characteristics of slow activation kinetics, at threshold potentials, fast inactivation and small unitary conductance (Perez-Reyes *et al*., 1998). Finally, recent evidence suggests that mibefradil (10 μM) may inhibit protein kinase C (PKC) activity in rat aortic vascular smooth muscle cells after activation by endothelin (Hermsmeyer & Miyagawa, 1996). This activity did not occur with amlodipine 10 μM but suggests that mibefradil at high concentration outside the therapeutic plasma concentration range may possess multiple actions that may contribute to vascular relaxation.

In conclusion, this study demonstrates in human tissue assays, the remarkable vascular to cardiac selectivity of a new tetralol class of T-type VOCC antagonist. This vascular selectivity and bradycardia with suppression of autonomic reflex activation would provide additional advantages for therapy of angina and hypertension.

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