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Relationship between the stereoselective negative inotropic effects of verapamil enantiomers and their binding to putative calcium channels in human heart

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1 Ventricular preparations from patients with mitral disease and hypertrophic obstructive cardiomyopathy (HOCM) were set up to contract isometrically. Ventricular membrane particles were also prepared and putative calcium channels were labelled with [^{3}H]-nimodipine.

2 Positive staircase was induced by varying the rate of stimulation of isolated strips from 6 min^{-1} to 120 min^{-1} in the presence of 6–60 μM (−)-adrenaline or (−)-noradrenaline. (−)-Verapamil 3–5 μM or (+)-verapamil 20–30 μM reversed the force-frequency relationship (i.e. caused negative staircase) in preparations from patients with mitral disease or HOCM.

3 In subendocardial strips of ventricular septum from 5 patients with HOCM paced at 60 min^{-1}, both (−)-verapamil and (+)-verapamil caused cardiodepression. Half-maximal cardiodepression was observed with 0.4 μM (−)-verapamil and with 3 μM (+)-verapamil.

4 [^{3}H]-nimodipine bound to ventricular membrane particles in a saturable, reversible fashion to a high affinity site with an equilibrium dissociation constant of 0.23 nM. The density of these sites was 95 fmol mg^{-1} of membrane protein. Binding of the tritiated 1,4-dihydropyridine was stereoselectively inhibited by 1,4-dihydropyridine enantiomers and nifedipine.

5 (−)-Verapamil and (+)-verapamil inhibited high affinity [^{3}H]-nimodipine binding in a negative heterotropic allosteric manner with (−)-verapamil being 5 times more potent than (+)-verapamil on an IC_{50} basis. At a given [^{3}H]-nimodipine concentration, (+)-verapamil inhibited a greater fraction of specific [^{3}H]-nimodipine binding.

6 The allosteric mode of (+)-verapamil inhibition of [^{3}H]-nimodipine binding was confirmed by kinetic studies. (−)-Verapamil shifted (+)-verapamil-binding inhibition curves to the right in an apparently competitive fashion.

7 The inversion of staircase caused by both verapamil enantiomers suggests that they cause a use-dependent channel blockade. The similar potency ratios for binding and for cardiodepression are indicative of a common locus of action for both verapamil enantiomers within the calcium channel.

Introduction

The force-frequency relationship (positive staircase) is inverted by racemic verapamil in rabbit ventricle (McCans *et al.*, 1974). This effect was confirmed by Chiba (1976, 1977) in dog ventricle and by Bayer *et al.* (1975a) in cat papillary muscle. Bayer *et al.* (1975b) also observed an inversion of the staircase with (−)-verapamil, but not with (+)-verapamil. They concluded that (−)-verapamil and (+)-verapamil interfered with excitation-contraction coupling at different sites.

Since qualitatively controversial results have been reported on verapamil enantiomers with respect to the force-frequency relationship and because (±)-verapamil is used in cardiovascular diseases, we decided to perform studies with human heart. Adrenaline enhances (Reuter & Scholz, 1977) but (±)-verapamil and (−)-verapamil decrease (Kohlhardt *et*

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al., 1972; Kohlhardt & Mnich, 1978; Ehara & Kaufmann, 1978) the slow inward current (carried mainly by calcium) of heart cells. As adrenaline and verapamil have functionally antagonistic effects (see also Fleckenstein *et al.*, 1967) and because the human heart is constantly exposed to catecholamines, we performed some of the experiments on tissues in the presence of (−)-adrenaline or (−)-noradrenaline. In order to test whether or not (−)-verapamil and (+)-verapamil act on the same site, we labelled membrane particles of human ventricle with [^{3}H]-nimodipine. This ligand of high specific activity has a high affinity for putative calcium channels in several tissues (Ferry & Glossmann, 1982a; Glossmann *et al.*, 1983a) including heart (Janis *et al.*, 1982; Ferry & Glossmann, 1983). We have examined the regulation of [^{3}H]-nimodipine binding by the verapamil enantiomers, in order to elucidate directly whether (−)-verapamil and (+)-verapamil bind to the same site(s).

Methods

Isolated tissues and patient material

Papillary muscles, trabeculae and strips were excised from the left ventricle of 5 patients suffering from combined mitral valve lesion. Four patients were female (48–68 years old); the fifth patient was a 52 year old male. Seven additional patients of either sex had hypertrophic obstructive cardiomyopathy (HOCM) with an age range of 34–52 years. The patients with mitral lesions had been treated with diuretics (thiazides) and digoxin for several years. None of the patients with mitral lesion had been treated with β-adrenoceptor blocking agents. The HOCM patient of Figure 1 had received (±)-verapamil orally 240 mg day^{-1} until 3 days before the operation. One HOCM patient was treated orally with (±)-propranolol 80 mg day^{-1} until the day before the operation. The 5 other HOCM patients had received neither a β-blocker nor verapamil 3 days prior to the operation.

Anaesthesia was with ethrane. Fentanyl was used for the induction of anaesthesia and pancuronium as a muscle relaxant. The patients with mitral lesion underwent replacement of the mitral valve. The patients with HOCM underwent partial ablation of ventricular septal tissue. The excised valve and adhering tissues or ventricular septal tissues were transported to the laboratory in a sealed vial containing oxygenated physiological solution at room temperature. The laboratory was built close to the operating theatre (Kaumann *et al.*, 1982), so that dissection and setting up of the tissues were started in less than 5 min after surgical removal. The physiological solution contained (mmol l^{-1}): Na^+ 140, K^+ 5, Ca^{2+} 2.25, Mg^{2+} 1, Cl^- 98.5, HCO_3^- 29, HPO_4^{2-} 1, fumarate 10, pyruvate 5, L-glutamate 5, glucose 10, disodium ethylenediamine tetraacetic acid (EDTA) 0.04, equilibrated with 95% O_2 and 5% CO_2. The water was deionized and double distilled in glass.

Thin papillary muscles and trabeculae (width < 1.2 mm) or strips (cut approximately 10×4 mm, width ~ 1 mm, without causing visible damage to the endocardial surface) of mitral patients were dissected as previously described (Kaumann *et al.*, 1982). The tissue of the HOCM patients consisted of a cuneiform resection of septum covered partially by endocardium. The tissues were set up in 50 ml organ baths (Blinks, 1965) containing the above solution. At least 3 tissues from each patient with mitral lesion were mounted in 3 different baths. Only one to 3 suitable tissues from each HOCM patient were studied. Experiments were carried out at 37°C. The muscles were attached to strain-gauge transducers. The tissues were driven at 0.2 Hz with square wave pulses of 5 ms duration and currents of just threshold intensity (less than 2 V delivered through the electrodes). The muscles were stretched to the length at which maximum peak tension developed.

In addition to blocking calcium channels, methoxyverapamil (D-600) has a moderate affinity for α-adrenoceptors (Fairhurst *et al.*, 1980). It is likely that verapamil also exhibits some affinity for α-adrenoceptors. Because adrenaline appears to interact with human atrial α-adrenoceptors (Wagner *et al.*, 1980) and to avoid possible α-adrenoceptor blockade by the enantiomers of verapamil, the strips were incubated for 2 h with phenoxybenzamine 5 μmol l^{-1}. After this period, phenoxybenzamine was washed out and not administered thereafter. This procedure causes irreversible blockade of myocardial α-adrenoceptors (Kaumann, 1970). In HOCM patients cumulative concentration-effect curves for (−)-verapamil and (+)-verapamil were determined in tissues (stimulated at 1 Hz) not treated with catecholamines.

The tissues were allowed to equilibrate at least 1 h after the washout of phenoxybenzamine. In order to demonstrate the staircase with catecholamines two successive concentration-effect curves for (−)-adrenaline or (−)-noradrenaline were determined cumulatively on each muscle with 1 h between curves. The second concentration-effect curve for a catecholamine was determined by using the highest concentrations of 6–60 μmol l^{-1}. Such concentrations of each catecholamine cause approximately 90% of the maximum inotropic effect as shown on human ventricular preparations (Kaumann *et al.*, 1981; 1982). The tissues were stimulated for approximately 3 min periods at 10, 5, 2, 1, and 0.5 s intervals. In tissues from patients with mitral lesion the following procedure was used: 15 min after the staircase (−)-verapamil 3 μmol l^{-1} was administered to one tissue and (+)-

verapamil 30 μmol l^{-1} to the second tissue. The third tissue did not receive verapamil; 30 min later the tissues were washed with warm solution and the catecholamine and the enantiomers of verapamil readministered immediately to the corresponding baths. The staircase was repeated 45 min after the first administration of verapamil enantiomers.

The concentrations of (−)-verapamil and (+)-verapamil were chosen assuming that the effects of verapamil are stereoselective. This assumption is supported by the finding that (−)-verapamil is 3 to 10 times more potent than (+)-verapamil in a variety of systems (Kaumann & Serur, 1975; Kaumann & Uchitel, 1976; Schwamborn *et al.*, 1976; Raschak, 1976).

Membrane particles

Human ventricular tissues were transported in the oxygenated physiological solution and dissected on ice. Membrane particles were prepared as described (Kaumann & Birnbaumer, 1974) as validated for human myocardium (Kaumann *et al.*, 1982) and stored at −80°C. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Binding assay

All binding experiments were performed under sodium lighting. Incubations were performed in a volume of 0.25 ml in Tris-HCl 50 mmol l^{-1} phenylmethylsulphonylfluoride (PMSF) 0.1 mmol l^{-1}, (−)-ascorbic acid 1 mmol l^{-1}, pH 7.4 at 37°C. The concentration of membrane protein was 0.16–0.32 mg ml^{-1}, and blanks were defined by unlabelled nimodipine 1 μmol l^{-1}. Following 30 min incubation at 37°C the assay mixture was diluted with 3.5 ml of ice-cold Tris-HCl 20 mmol l^{-1}, $MgCl_2$ 10 mmol l^{-1} polyethylene glycol 6000 10% w/v (filtration medium) and filtered through a 3.5 cm Whatman GF/C filter under strong suction. The filter was washed twice with buffer, dried and counted for radioactivity by liquid scintillation counting. It has previously been shown, that only 50% of the chiral 1,4-dihydropyridine [^{3}H]-nimodipine is bound by addition of excess receptors (Ferry & Glossmann, 1982b) and that the 4S (−)-enantiomer is more potent than the 4R (+)-enantiomer nimodipine as a slow channel blocker in functional tests (Towart *et al.*, 1981). Therefore in all calculations only the bindable enantiomer of the racemic [^{3}H]-nimodipine employed has been taken into account.

Kinetic experiments

Association reactions were started by addition of 0.05 ml of [^{3}H]-nimodipine to 0.2 ml of membranes pre-incubated either in the presence or absence of unlabelled nimodipine 1 μmol l^{-1} and incubated for up to 30 min prior to the separation of bound and free ligand.

For dissociation reactions 0.01 ml of nimodipine 25 μmol l^{-1}, or 0.01 ml nimodipine 25 μmol l^{-1} plus (+)-verapamil 2.5 mmol l^{-1} was added to 0.25 ml of [^{3}H]-nimodipine-channel complexes for times up to 5 min before separation of bound and free ligand.

Computer fitting of binding data

Association reactions were fitted to the differential form of the second order rate equation describing a biomolecular reaction by the least squares method (DeLean *et al.*, 1978). The equation used is:

$$RL/dt = k_{+1}(R - RL)(L - RL) - k_{-1}(RL)$$

Where RL is [^{3}H]-nimodipine receptor-complex concentration, R is the receptor concentration, L is the free, bindable [^{3}H]-nimodipine concentration, k_{+1} is the association-rate constant and k_{-1} is the dissociation-rate constant. Equilibrium dissociation constants K_D and the density of saturable binding sites (B_{max}) were calculated by assuming mass-law interaction of [^{3}H]-nimodipine with the Ca^{2+}-channel receptor site (DeLean *et al.*, 1978).

Binding inhibition data were fitted to the general dose-response equation (Ferry & Glossmann, 1982b). All fits were done with the BMDP biomedical statistics package on a CYBER 172 computer. Means were compared with Student's two tailed t test, $P < 0.05$ being the accepted level of significance.

Drugs

(−)-Noradrenaline bitartrate monohydrate and (−)-isoprenaline bitartrate were from Sterling-Winthrop (Renslaer, N.Y., U.S.A.), (−)-adrenaline bitartrate was from Serva (Heidelberg, F.R.G.). Stock solutions (100 mmol l^{-1}) of catecholamines were made in deionized, redistilled water containing EDTA 0.04 mmol l^{-1} and adjusted to pH 4 with HCl. Dilutions of catecholamines were prepared in EDTA 0.04 mmol l^{-1}. Phenoxybenzamine hydrochloride was from Smith, Kline & French. (−)-Verapamil hydrochloride and (+)-verapamil hydrochloride were from Knoll-BASF (Ludwigshafen, F.R.G.) or from Hoffmann-La Roche (Basle, Switzerland). We tested both batches of enantiomers on isolated tissues (cardiodepression) and membranes (binding experiments). Cardiodepressant potencies and binding characteristics were the same for both batches of the enantiomers.

[^{3}H]-nimodipine (145–150 Ci $mmol^{-1}$, radiochemical purity $>$ 99%), unlabelled nimodipine and nifedipine were from Bayer AG. (Wuppertal, F.R.G.). Optically pure enantiomers of PN 200–110

(isopropyl-4-(2,1,3-benzoxadiazol-4-yl) -1,4-dihydro-2,6-dimethyl-5-methoxycarbonyl-pyridine-3-carboxylate) were from Sandoz AG (Basle, Switzerland). The 1,4-dihydropyridines were dissolved in dimethylsulphoxide (DMSO) at 10 mmol l^{-1} and stored at − 20°C in the absence of u.v.-light. (+)-*cis*-Diltiazem was from Gödecke (Freiburg, F.R.G.).

Results

Stereoselective inversion of the staircase by (−)-verapamil and (+)-verapamil

Increasing the beating frequency from 6 min^{-1} to 120 min^{-1} augmented the contractile strength of (−)-adrenaline treated muscles from HOCM patients (Figure 1) and patients with mitral lesions (Figure 3). A similar positive frequency-force relationship was observed with 4 (−)-noradrenaline treated muscles from patients with mitral lesions (experiments not shown). This positive staircase pattern was not significantly different when two successive runs of the staircase procedure were determined 1 h apart (Figure 3a).

Both (−)-verapamil (Figures 2, 3) and (+)-verapamil (Figures 1, 2, 3) caused reductions of contractile strength of (−)-adrenaline treated preparations. The effect was more pronounced the higher the driving frequency. Thus, both (+)-verapamil and (−)-verapamil cause a negative staircase; the effect was observed in preparations from both patients with mitral lesion (Figures 2, 3) and patients with HOCM (Figure 1). The inversion of staircase became already apparent after 10 min incubation with (−)-verapamil 5 μmol l^{-1}. The negative stiarcase induced by both isomers of verapamil was also observed in two muscles (mitral lesion) treated with (−)-noradrenaline (experiments not shown). In septal strips of 5 HOCM patients paced at 60 min^{-1}, (−)-verapamil was 8 times more potent than (+)-verapamil as a cardiodepressant. Log EC_{50}-values

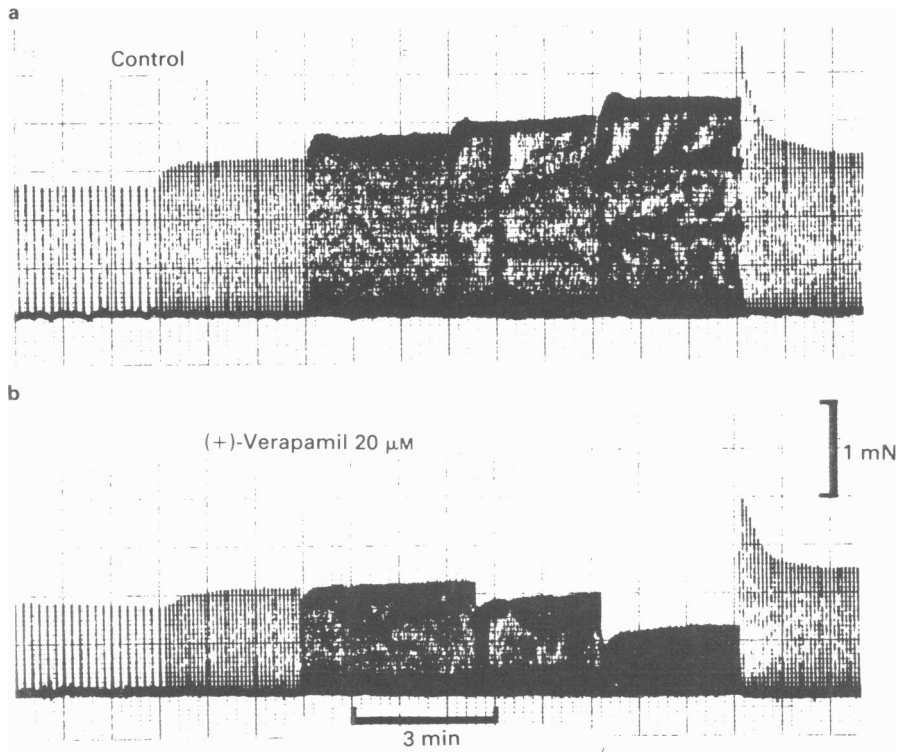

Figure 1 Positive staircase (a) and negative staircase caused by 45 min incubation with (+)-verapamil 20 μmol l^{-1} (b) Septal ventricular strip from a 48 year old male with hypertrophic obstructive cardiomyopathy. The strip was exposed to (−)-adrenaline 20 μmol l^{-1} before and during the staircase procedures. The muscle was stimulated (from left to right) to cause 6, 12, 30, 60 and 120 contractions min^{-1} and then left at 12 contractions min^{-1}.

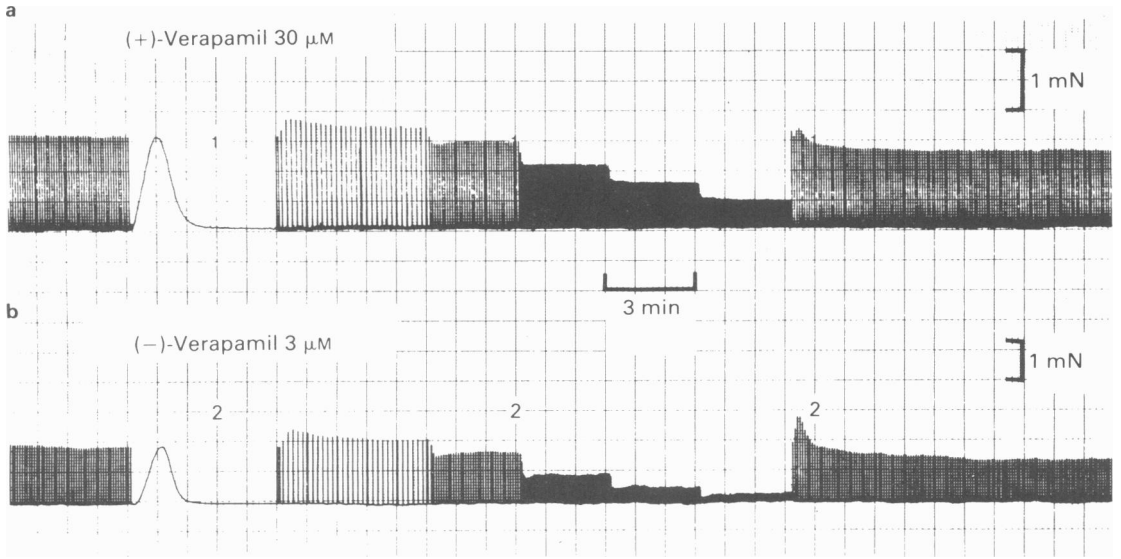

Figure 2 Negative staircase caused by 45 min incubations with (+)-verapamil 30 μmol l^{-1} (a) or with (−)-verapamil 3 μmol l^{-1} (b). Left ventricular strips of a 68 year old female with mitral lesion. The strips were exposed to (−)-adrenaline 60 μmol l^{-1} during the staircase procedure and incubation with enantiomers of verapamil. On the left where contractions are 12 min^{-1}, a fast speed tracing (50 mm s^{-1}) was obtained. Two contractions later the muscles were stimulated to cause 6, 12, 30, 60 and 120 contractions min^{-1} and then left at 12 min^{-1}.

(± s.e.mean) were 6.4 ± 0.2 for (−)-verapamil and 5.5 ± 0.2 for (+)-verapamil.

Basic properties of [^{3}H]-nimodipine binding to human heart membranes

High affinity [^{3}H]-nimodipine binding to human heart was linear with membrane protein over the range 0.04 to 0.80 mg ml^{-1} of membrane protein and was not affected by DMSO (the solvent used for unlabelled drugs) up to 10% volume/volume. The signal to noise ratio at a K_D concentration (~0.25 nmol l^{-1}) of [^{3}H]-nimodipine employing ~60 μg of membrane protein per tube in a volume of 0.25 ml was in the range 2–4. Because most of the blank binding constituted filter blank the use of higher protein concentrations (i.e. > 30 μg/tube) was important. However, because of the limited human material available this means that the muscle excised from one patient allows the performance of only 50 to 200 assay points. As we have been assaying adrenoceptors in these membranes (Kaumann *et al.*, in preparation) for which some of the tested drugs oxidize easily we added ascorbic acid 1 mmol l^{-1} to incubations which has no effect on specific [^{3}H]-nimodipine binding up to 5 mmol l^{-1}. [^{3}H]-nimodipine binding is divalent cation-dependent in heart (Glossmann *et al.*, 1983a). EDTA 5 mmol l^{-1} caused a 50% decrease in high affinity [^{3}H]-nimodipine binding in human heart membranes (not shown) but exogenously added Ca^{2+} up to 5 mmol l^{-1} had no effect, suggesting that there is sufficient endogenous Ca^{2+} to fulfil the divalent cation requirement for [^{3}H]-nimodipine binding. (+)-*cis*-Diltiazem stimulated specific [^{3}H]-nimodipine binding with an EC_{50} of 1–2 μmol l^{-1}, but (−)-*cis*-diltiazem was without effect (not shown). In human cardiac membranes diltiazem disasteroisomers at concentrations of 10 μmol l^{-1} increased blank binding of [^{3}H]-nimodipine (Figure 4). This we have never observed in guinea-pig brain, heart or skeletal membranes (Ferry & Glossmann, 1983). Therefore, when the effects of (+)-*cis*-diltiazem were evaluated both total and blank binding (blanks in the presence of nimodipine 1 μmol l^{-1}) were always performed. The effect of 10 μmol l^{-1} (+)-*cis*-diltiazem on [^{3}H]-nimodipine blank binding can be seen in Figure 4.

Saturation analysis of [^{3}H]-nimodipine binding

[^{3}H]-nimodipine binds in a saturable manner with high affinity to human heart membranes at 37°C. Figure 5 shows the saturation isotherm of [^{3}H]-nimodipine

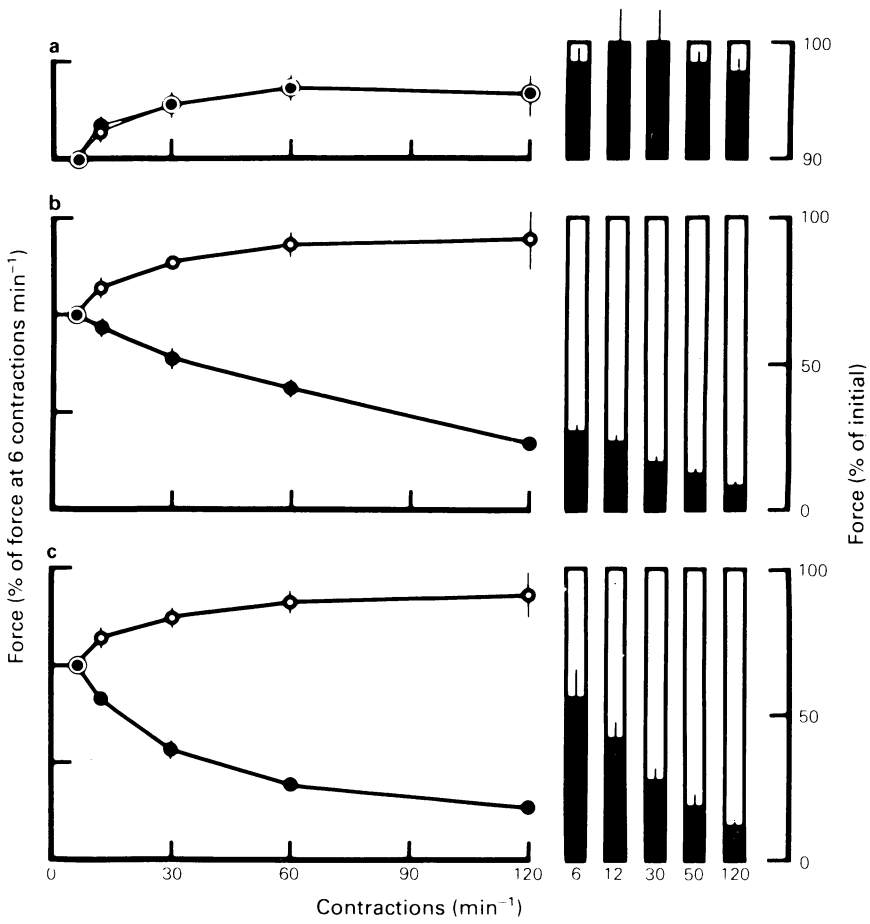

Figure 3 Inversion of staircase by (+)-verapamil and (−)-verapamil. Ventricular preparations from mitral lesions exposed to (−)-adrenaline 60 μmol l^{-1}. Two successive staircases were determined; 60 min after the first staircase (○) a second staircase (●) was determined in the absence (a) or presence of (+)-verapamil 30 μmol l^{-1} (b) or (−)-verapamil 3 μmol l^{-1} (c). Histograms show the negative inotropic effects of (+)-verapamil (b) and (−)-verapamil (c) at different beating frequencies. Stippled columns represent the peak tension at each beating frequency of the second staircase. Bars through symbols and histograms are s.e.mean.

from 5 independent experiments, each with membranes from different patients. Clearly even between patients the binding capacity (B_{max}) is very consistent with a range of 77–110 fmol l mg^{-1} protein and an average value of 95 ± 10 fmol mg^{-1} of protein. The K_D of [^{3}H]-nimodipine is 0.23 ± 0.01 nmol l^{-1} (n = 5) from the 5 patients shown in Figure 4. In saturation isotherms the blank binding at a K_D concentration of [^{3}H]-nimodipine is 20–35% of total filter-retained radioactivity. (+)-*cis*-Diltiazem increases the B_{max} of [^{3}H]-nimodipine high affinity sites without changing the K_D (Figure 5) as has been previously reported in guinea-pig heart membranes (Ferry & Glossmann, 1983). In three patients where we had sufficient membranes to perform full saturation isotherms in the presence and absence of (+)-*cis*-diltiazem the B_{max} in the absence of (+)-*cis*-diltiazem was 107 ± 5.5 fmol mg^{-1} (n = 3, mean ± s.e.mean) of protein with a K_D-value of 0.32 ± 0.06 nmol l^{-1} and with (+)-*cis*-diltiazem the B_{max} was increased to 132 ± 7.5 fmol mg^{-1} protein ($P < 0.01$) with a K_D of 0.4 ± 0.13 nmol l^{-1}. Thus, on average, 10 μmol l^{-1} (+)-*cis*-diltiazem in-

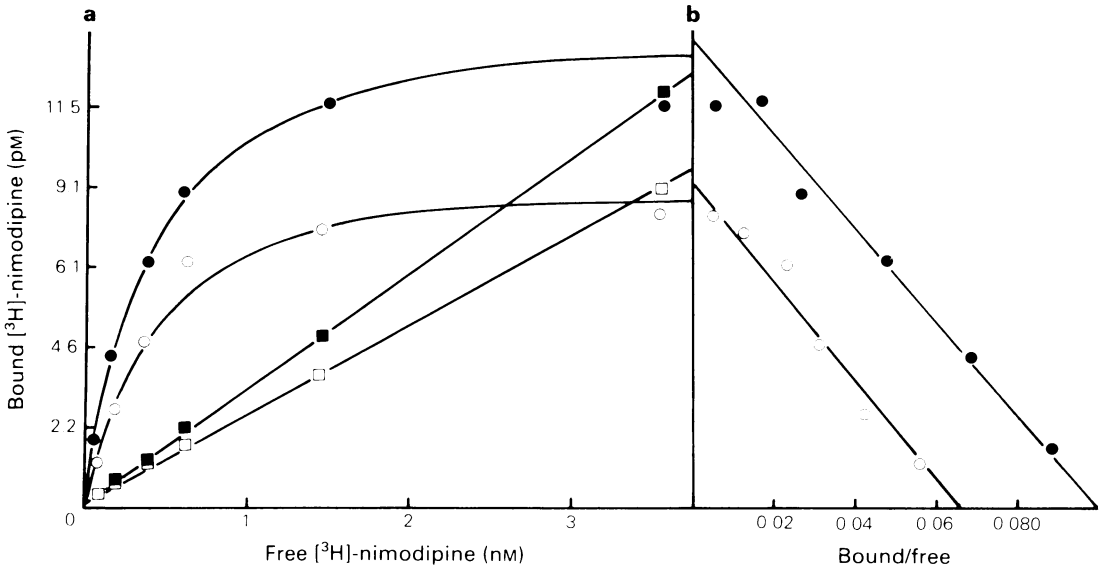

Figure 4 Stimulation of [^{3}H]-nimodipine by (+)-*cis*-diltiazem: (a) shows the specific (circles) and blank (squares) in the presence (filled symbols) and absence (open symbols) of (+)-*cis*-diltiazem 10 μmol l^{-1}. Both saturations were performed with membranes from the same HOCM patient in parallel, with 23 μg of membrane protein in a volume of 0.25 ml with a concentration range of 23 to 3560 pmol l^{-1} free [^{3}H]-nimodipine. (b) Hofstee plots of the specific binding shown in (a). In the absence of (+)-*cis*-diltiazem the K_D was 0.28 nmol l^{-1} and B_{max} 100 fmol mg^{-1} of protein and in the presence of 10 μmol l^{-1} (+)-*cis*-diltiazem the K_D was 0.23 nmol l^{-1} and B_{max} 132 fmol mg^{-1} of protein.

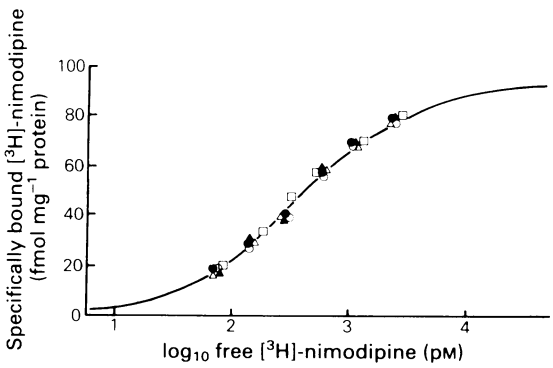

Figure 5 Saturation of [^{3}H]-nimodipine binding in human heart membranes. The data shown are for 3 HOCM and 2 mitral valve patients. Blanks defined by unlabelled nimodipine 1 μmol l^{-1} have been subtracted and each point is the mean value of duplicate determinations. The saturation isotherms were fitted by non-linear regression analysis. The values for three HOCM patients with B_{max} in fmol mg^{-1} protein and K_D in pmol l^{-1}, respectively, are (○) 110, 256; (□) 85, 112; (●) 102, 281 and for the two mitral patients, (△) 99, 236 and (▲) 91, 247.

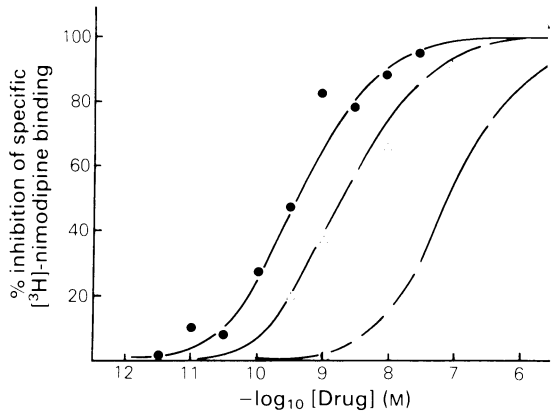

Figure 6 Inhibition of [^{3}H]-nimodipine binding by 1,4-dihydropyridines. Binding inhibition profiles of 1,4-dihydropyridines. The values are means of duplicate determinations. The [^{3}H]-nimodipine concentration was 460 pmol l^{-1}, the heart membrane protein concentration 85 μg in a total volume of 0.25 ml. Total binding was 1690 d.p.m. per filter and blank binding 600 d.p.m. per filter. The K_i values were (+)-PN 200-110 (●) 0.2 nmol l^{-1}, (−)-PN 200-110 (○) 24 nmol l^{-1} and nifedipine (△) 0.9 nmol l^{-1}. The experiment is representative of three similar experiments on HOCM patients for each drug.

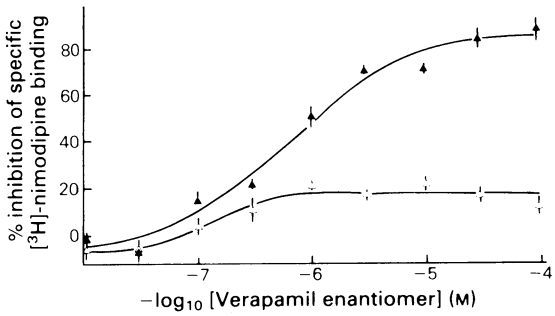

Figure 7 Inhibition of [^{3}H]-nimodipine binding by verapamil enantiomers. (−)-Verapamil (Δ)- and (+)-verapamil (▲)-inhibition curves from four independent experiments performed on four different HOCM patients with [^{3}H]-nimodipine concentrations of ~0.65 nmol l^{-1} and protein concentrations of 40 to 80 μg in 0.25 ml. Values are means of values determined in duplicates with s.e.mean shown by vertical lines. The pooled data were fitted to the general dose-response equation and gave the following parameter estimates (asymptotic s.d.): for (−)-verapamil, IC_{50} = 123 ± 53 nmol l^{-1}, maximum inhibition 17.6 ± 1.9% and slope factor 1.6 ± 1.0; for (+)-verapamil, IC_{50} = 630 ± 190 nmol l^{-1}, maximum inhibition 87 ± 6% and slope factor 0.83 ± 0.22. The % inhibition refers to the % of specific binding displaced.

creased the density of high affinity [^{3}H]-nimodipine binding sites by 23 ± 5% in the three HOCM patients examined.

Binding profile of high affinity [^{3}H]-nimodipine sites

Specific high affinity [^{3}H]-nimodipine binding is inhibited stereoselectively by the optically pure enantiomers of the benzoxadiazol 1,4-dihydropyridine PN 200-110, the (+)-enantiomer being 120 fold more potent than the (−)-enantiomer (Figure 6) (+)-PN 200-110 is 5 fold more potent than nifedipine (Figure 6). The phenylalkylamine calcium antagonists (−)- and (+)-verapamil which are chemically unrelated to 1,4-dihydropyridines also inhibit [^{3}H]-nimodipine binding. The inhibition, using ~2 K_D concentrations of [^{3}H]-nimodipine, is incomplete for (−)-verapamil (Figure 7) and nearly complete for (+)-verapamil. However, (−)-verapamil is more potent than (+)-verapamil on an IC_{50} basis for the binding which is inhibited. When (+)-verapamil and (−)-verapamil binding inhibition curves were performed in the presence of increasing concentrations of [^{3}H]-nimodipine the alterations of the binding inhibition curve were not compatible with [^{3}H]-nimodipine and verapamil enantiomers binding to the same drug receptor site (Figure 8). Isoprenaline at 0.1 mmol l^{-1}

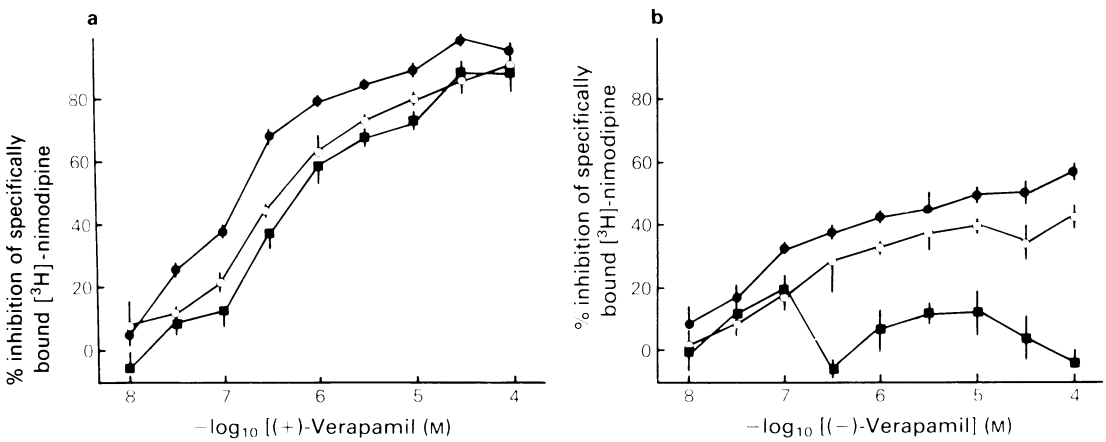

Figure 8 Binding inhibition of verapamil enantiomers at different [^{3}H]-nimodipine concentrations. Points are triplicate determinations of a single representative experiment on membranes from a HOCM patient; vertical lines show s.d. For the (+)-verapamil (a) experiment the protein concentration was 59.5 μg in 0.25 ml. The [^{3}H]-nimodipine concentrations employed with total (t) and blank (B) binding d.p.m. ± s.d. for n = 3 determinations were (■) 1.9 nmol l^{-1}, t = 6224 ± 276, B = 3101 ± 183; (○) 1.1 nmol l^{-1}, t = 4954 ± 83, B = 1997 ± 49; (●) 0.28 nmol l^{-1}, t = 2276 ± 120, B = 685 ± 71. For the (−)-verapamil experiment (b) 60.3 μg of protein in 0.25 ml was employed. At a [^{3}H]-nimodipine concentration of (■) 2.54 nmol l^{-1}, t = 6064 ± 418, B = 2578 ± 156; 0.84 nmol l^{-1} (○) t = 3254 ± 284, B = 820 ± 171 and at 0.32 nmol l^{-1} (●) t = 1882 ± 141, B = 301 ± 25.

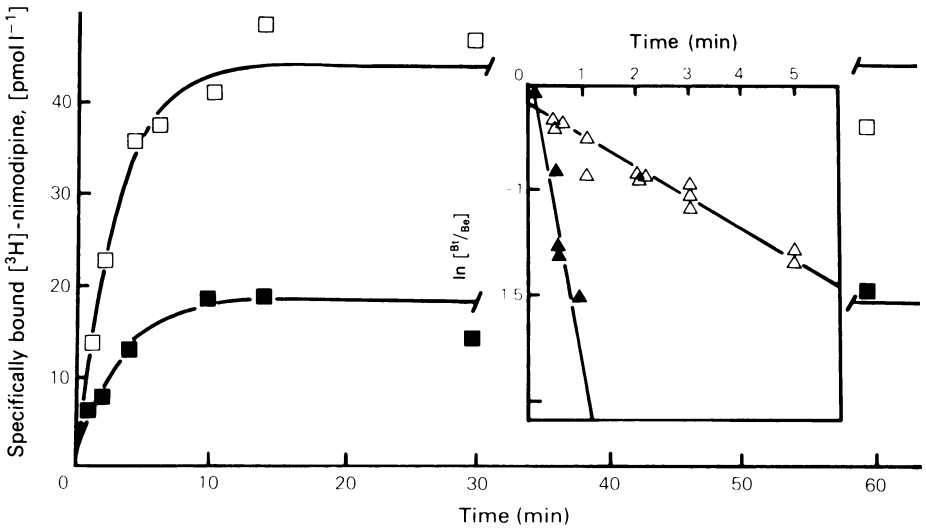

Figure 9 Kinetics of [^{3}H]-nimodipine binding. Association of [^{3}H]-nimodipine with cardiac membranes (HOCM patient) in the absence (□) and presence (■) of (+)-verapamil 300 nmol l^{-1}; 150 μg of membrane protein was employed in an assay volume and at zero time the free concentration of [^{3}H]-nimodipine was 320 pmol l^{-1}. The points are means of duplicates from which blank binding has been subtracted at equilibrium in the absence of (+)-verapamil the total binding was (mean ± s.d.) 5116 ± 538 d.p.m. and in the presence of (+)-verapamil 300 nmol l^{-1} was 2913 ± 404 d.p.m. Under both conditions blank binding was instantaneous and stable at ~ 1400 d.p.m. The specific binding was fitted to the differential form of the second order rate equation, the parameter estimates in the absence of (+)-verapamil (± asymptotic standard deviation) were $k_{+1} = 0.82 \pm 0.11$ (nmol/l)$^{-1}$ min^{-1} and $k_{-1} = 0.40 \pm 0.07$ min^{-1}, in the presence of (+)-verapamil 300 nmol l^{-1}, $k_{+1} = 0.28 \pm 0.06$ (nmol/l)$^{-1}$ min^{-1} and $k_{-1} = 0.5 \pm 0.13$ min^{-1}. The inset shows the dissociation of [^{3}H]-nimodipine from channel complexes. Be is equilibrium specific [^{3}H]-nimodipine binding, Bt is specifically bound [^{3}H]-nimodipine t min after addition of nimodipine 1 μmol l^{-1} (△) or a mixture of nimodipine 1 μmol l^{-1} with (+)-verapamil 100 μmol l^{-1} (▲). Each point is the mean of a duplicate determination and points have been pooled from $n = 3$ independent experiments. The lines have been drawn by linear regression analysis. The k_{-1} induced by nimodipine is 0.37 ± 0.07 min^{-1} (mean ± s.e.mean, $n = 3$) and by the nimodipine/(+)-verapamil mixture 2.66 ± 0.44 min^{-1} (mean ± s.e.mean, $n = 3$).

did not change (−)- and (+)-verapamil binding inhibition curves of [^{3}H]-nimodipine or [^{3}H]-nimodipine steady-state binding (not shown).

Kinetics of [^{3}H]-nimodipine binding

The complex form of inhibition by (+)-verapamil has been examined in detail with kinetic experiments (Figure 9); (+)-verapamil 300 nmol l^{-1} caused an approximately 3 fold decrease in the association rate constant from 0.82 ± 0.11 min^{-1} (nmol l)$^{-1}$ to 0.28 ± 0.06 min^{-1} (nmol l)$^{-1}$. When the high affinity [^{3}H]-nimodipine receptor complex was dissociated by addition of 1 μmol l^{-1} unlabelled nimodipine the dissociation rate constant (k_{-1}) was 0.37 ± 0.07 min^{-1}. This is very close to the k_{-1} of [^{3}H]-nimodipine calculated by fitting the association reaction data to the differential form of the second order rate equation (0.4 ± 0.07 min^{-1}, see Figure 9). When a mixture of nimodipine 1 μmol l^{-1} and (+)-verapamil 100 μmol l^{-1} was used to block the association reaction, dissociation was accelerated 7 fold to a k_{-1} of 2.66 ± 0.4 min^{-1} (Figure 9).

Interaction of (−)- and (+)-verapamil

In order to show that (−)-verapamil and (+)-verapamil may act on the same drug receptor site, (+)-verapamil binding inhibition curves were performed in the presence of increasing concentrations of (−)-verapamil which shift the (+)-verapamil binding inhibition curve to the right (Figure 10). The slope of the binding inhibition curves of (+)-verapamil became somewhat steeper with increasing concentrations of (−)-verapamil precluding precise analysis of the interaction of the verapamil enantiomers with phenylakylamine drug receptor sites. As a first approximation, however, we estimated (Gaddum,

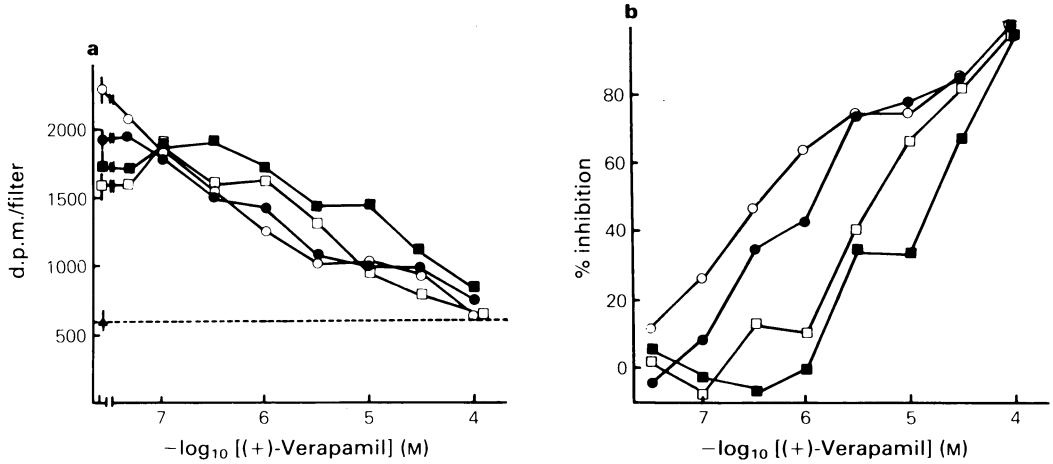

Figure 10 Interaction of verapamil enantiomers. (+)-Verapamil inhibition of binding in the absence (○) and presence of 0.1 μmol l^{-1} (●), 1 μmol l^{-1} (□) and 10 μmol l^{-1} (■) (−)-verapamil: (a) shows the data in original form. [^{3}H]-nimodipine 410 pmol l^{-1} was incubated with 55 μg of the membrane protein in a volume of 0.25 ml. Binding in the absence of (+)-verapamil are means from four determinations (s.d. shown by vertical lines), other points were determined in duplicates. The dashed line shows the non-specific binding. (b) Shows a transformation of data from (a). The (+)-verapamil binding inhibition curves were normalized by defining the bound d.p.m. with (+)-verapamil 100 μmol l^{-1} to be 100% inhibition. Hill plots of the data gave the following parameter estimates in the absence of (−)-verapamil, IC_{50} = 690 nmol l^{-1}, nH = 0.50, with 0.1 μmol l^{-1} (−)-verapamil, IC_{50} = 1500 nmol l^{-1}, nH = 0.67, with 1 μmol l^{-1} (−)-verapamil, IC_{50} = 5000 nmol l^{-1}, nH = 0.82. Data are from one HOCM patient. Similar data were obtained with membranes from another HOCM patient.

1937) from the dose-ratios at IC_{50} that the overall equilibrium dissociation constant is ~85 nmol l^{-1} for (−)-verapamil.

Discussion

Uniform mode of action of (−)-verapamil and (+)-verapamil

The present experiments confirm that both (−)-verapamil and (+)-verapamil invert the positive staircase to a negative staircase in human myocardium as has been previously reported for (±)-verapamil in rabbit, cat and dog ventricular preparations (McCans *et al.*, 1974; Bayer *et al.*, 1975a; Chiba 1976; 1977). In cat papillary muscle Bayer *et al.* (1975b) observed the staircase inversion only with (−)-verapamil, but not with (+)-verapamil. Bayer *et al.* (1975b) therefore attributed the inversion of the staircase by racemic verapamil solely to the (−)-enantiomer, and concluded that the actions of (+)-verapamil were due to blockade of Na^+-channels. On the other hand, Nawrath *et al.* (1981) reported for cat papillary muscle that both (−)-verapamil and (+)-verapamil cause negative inotropic effects that are qualitatively identical. As found for human ventricular muscle, (−)-verapamil was more potent than (+)-verapamil in the experiments of Nawrath *et al.* (1981) on cat papillary muscle and the negative inotropic potency of (+)-verapamil is the same in both species (IC_{50} ≈ 3 μmol l^{-1}). By measuring the maximum rate of rise (*dV/dt max*) of the action potential as an indication of availability of the Na^+-channels, Nawrath *et al.* (1981) also showed that only very high concentrations of both verapamil enantiomers (> 10 μmol l^{-1}) decreased *dV/dt max*. Because the concentrations of (+)-verapamil causing negative inotropic effects are lower than those decreasing *dV/dt max* it seems unlikely that reduced availability of Na^+-channels is involved. A basic interpretation of the negative staircase produced by verapamil is probably related to the observation that (−)-verapamil reduces the slow inward clacium current to a greater extent the higher the beating frequency is (Ehara & Kaufmann, 1978). This frequency-dependent effect was also observed with methoxyverapamil (D-600) (McDonald *et al.*, 1980). D-600 and verapamil have little effect if tissues are not stimulated with relatively high frequency (Kaumann & Uchitel, 1976; Ehara & Kaufmann,

1978; McDonald *et al.*, 1980). McDonald *et al.* (1980) suggested that the frequency-dependent reduction of the slow inward calcium current is use- and voltage-dependent. Thus, a greater decrease in contractile strength is expected with verapamil the higher the beating frequency, which was indeed observed in human ventricle (see also Kaumann & Uchitel, 1976, for this interpretation). Because we could not find qualitative differences for the pattern of cardiodepression for (−)-verapamil and (+)-verapamil it appears that the cardiodepressant action not only of (−)-verapamil but also of (+)-verapamil has similar use-dependency.

To examine the possible interaction of verapamil with the slow calcium channel more directly, we performed labelling experiments with the 1,4-dihydropyridine [^{3}H]-nimodipine. Our equilibrium dissociation constant K_D of 0.2–0.4 nmol l^{-1} for [^{3}H]-nimodipine binding in human ventricle agrees well with reported constants for the hearts of other species. Thus, heart K_D-values were found to be 0.25 nmol l^{-1} for the calf (Glossmann *et al.*, 1983a), 0.26 nmol l^{-1} for guinea-pig (Ferry & Glossmann, 1983) and 0.24 to 1.0 nmol l^{-1} for rat (Janis *et al.*, 1982; De Pover *et al.*, 1983b). Our K_D-value of nifedipine (as a competitor for [^{3}H]-nimodipine binding) of 1 nmol l^{-1} agrees well with a K_D of 1.8 nmol l^{-1} reported for [^{3}H]-nifedipine on rabbit heart reported by Holck *et al.* (1982). Furthermore, our stereoselectivity ratio of 120 for human ventricle for the enantiomers of the 1,4-dihydropyridine PN 200-110 agrees with a similar ratio reported by Ferry & Glossmann (1982b) for the putative calcium channels of the skeletal muscle t-tubulus. Finally, (+)-*cis*-diltiazem increases the density of [^{3}H]-nimodipine high affinity binding sites by 21% in guinea-pig heart (Ferry & Glossmann, 1983) and 23% in human heart (present work, Figure 5) without changing the equilibrium dissociation constant. The stimulation of [^{3}H]-nimodipine binding by (+)-*cis*-diltiazem complicates the determination of the maximal density of high affinity binding sites. Previously it has been suggested that the binding site can exist in two states, with low and high affinity for [^{3}H]-nimodipine and that (+)-*cis*-diltiazem increases the proportion of sites in high affinity for [^{3}H]-nimodipine both in skeletal muscle (Ferry & Glossmann, 1982b) and heart (Ferry & Glossmann, 1983).

The close agreement for the hearts of man and other species of the binding characteristics of [^{3}H]-nimodipine and the high stereoselectivity of the (+)-*cis*-diltiazem effect as well as the binding constant of nifedipine is consistent with labelling of the same loci. A variety of arguments support the view that these loci are a component of the slow calcium channels, i.e. a correlation has been reported between binding inhibition and cardiodepression by an extensive series of chemically heterogeneous calcium antagonists (Holck *et al.*, 1982). Further, in the purified t-tubule of skeletal muscle the density of binding sites and the calcium channel density have been estimated to agree (Glossmann *et al.*, 1983b), and the enhanced [^{3}H]-nimodipine binding caused by (+)-*cis*-diltiazem correlates with a potentiation by (+)-*cis*-diltiazem of the cardiodepressant effects of nimodipine (De Pover *et al.*, 1983a).

Verapamil enantiomers inhibit high-affinity [^{3}H]-nimodipine binding in a complex non-competitive fashion. At ~0.7 nmol l^{-1} [^{3}H]-nimodipine, (−)-verapamil at 100 μmol l^{-1} inhibits 20% of [^{3}H]-nimodipine binding, whereas (+)-verapamil inhibits nearly all high-affinity [^{3}H]-nimodipine binding. If (+)-verapamil were to compete with [^{3}H]-nimodipine for the same site, saturating concentrations of both, unlabelled nimodipine and (+)-verapamil should cause dissociation of [^{3}H]-nimodipine from its site with the same rate constant. However, co-administration of (+)-verapamil and nimodipine induced a dissociation of [^{3}H]-nimodipine which was 7 times faster than the dissociation caused by 1 μmol l^{-1} unlabelled nimodipine alone. (+)-Verapamil also decreased the rate of association 3 fold. Therefore, (+)-verapamil appears to decrease [^{3}H]-nimodipine binding by binding to a drug receptor site allosterically coupled to the 1,4-dihydropyridine site. As the dissociation constant is defined by the rate constants it can be calculated that (+)-verapamil causes (i.e. when bound to the channel) a 20 fold decrease in affinity of [^{3}H]-nimodipine binding.

In support of the detailed kinetic experiments the inhibition of [^{3}H]-nimodipine binding by (+)-verapamil and (−)-verapamil (Figure 8) as a function of [^{3}H]-nimodipine concentration does not follow a pattern predicted from competition of [^{3}H]-nimodipine and verapamil enantiomers at the same site. Therefore, it also appears from this combined evidence that (−)- and (+)-verapamil allosterically modulate [^{3}H]-nimodipine binding.

Experiments in smooth muscle preparations suggest that (±)-verapamil and the 1,4-dihydropyridine, (±)-nitrendipine, compete for a calcium binding site which may be located on the calcium channel site (Humphrey & Robertson, 1983). These authors performed experiments combining both drugs. They concluded that a common receptor (Paton & Rang, 1965) explained the data best. However, as we have shown using human heart membranes, and as shown by Bolger *et al.* (1983) on smooth muscle membranes and by Ferry & Glossmann (1982a) and by Murphy *et al.* (1983) in brain membranes, phenylalkylamines and 1,4-dihydropyridines bind to distinct drug receptors which are allosterically coupled. (±)-Verapamil appears to be 100 times more potent as a smooth muscle calcium antagonist (Humphrey & Robertson, 1983) and ~10 times more potent as an inhibitor of [^{3}H]-1,4-

dihydropyridine binding in smooth muscle (Bolger *et al.*, 1983) than both verapamil-enantiomers on heart muscle (this paper). The discrepancies in the potency of verapamil may imply tissue-specific heretogeneity of slow calcium channels. This has previously been suggested because of the tissue but not species specific regulation of [^{3}H]-nimodipine binding by (+)-*cis*-diltiazem (Ferry & Glossmann, 1983).

We conclude that (−)-verapamil and (+)-verapamil bind to a common (Figure 10) drug receptor site of the putative calcium channel. This conclusion is in line with the qualitatively similar pattern for cardiodepression (i.e. staircase inversion). Further, both the stereoselectivity ratios for binding (5) and cardiodepression (8) are similar, the (−)-enantiomer being more potent for both systems. This agreement may be indicative of a causal relationship. Labelling of the phenylalkylamine drug receptor sites which are allosterically coupled to the 1,4-dihydropyridine drug receptors has so far only been successful in skeletal muscle membranes with [^{3}H]-(±)-verapamil (Galizzi *et al.*, 1984; Goll *et al.*, 1984) and in hippocampus membranes with [^{3}H]-(−)-desmethoxyverapamil (Ferry *et al.*, 1984). Development of appropriate radioligands for the phenylakylamine drug receptor in heart membranes will allow a more direct test of the hypothesis that both verapamil enantiomers bind to and therefore probably act at a common receptor site associated with the calcium channel.

Clinical implications

Both, (−)-verapamil and (+)-verapamil, cause qualitatively similar haemodynamic, antiarrhythmic and electrocardiographic effects in the dog (Kaumann & Serur, 1975; Raschack, 1976) and cardiodepressant effects in human ventricle (this paper). (+)- Verapamil is merely 3 to 10 times less potent than (−)-verapamil. In humans two to three times higher verapamil plasma levels are necessary after oral intake in order to cause equieffective increases in P-R intervals of electrocardiograms as after intravenous administration (Eichelbaum *et al.*, 1980). The cause of this difference is that the bioavailability of (−)-verapamil is smaller than that of (+)-verapamil (Eichelbaum *et al.*, 1984) due at least in part to stereoselective metabolism in the liver and therefore the less potent (+)-verapamil can contribute significantly to the electrocardiographic effects of racemic verapamil especially after oral as opposed to intravenous administration. (+)-Verapamil is 8 times less effective than (−)-verapamil in causing cardiodepression of human ventricular preparations paced at 60 min^{-1}. Because of higher protein binding of (−)-verapamil (Eichelbaum *et al.*, 1984) and also because of a higher clearance of (−)-verapamil through the liver (Eichelbaum *et al.*, 1981) the bioavailability of (+)-verapamil is at least twice that of (−)-verapamil. Thus, it seems reasonable to conclude that at least 25% of the myocardial effects of orally administered racemic verapamil can be accounted for by the (+)-enantiomer in man.

Patients with hypertrophic obstructive cardiomyopathy (HOCM) appear to benefit from verapamil treatment (Kaltenbach *et al.*, 1976; Rosing *et al.*, 1979). The benefit with verapamil may become manifest due to the simultaneous occurrence of tachycardia and decrease of ventricular contractile strength in stressful situations. Our experiments are consistent with this idea because both enantiomers cause negative staircase in ventricular preparations of HOCM patients and both enantiomers appear to bind to a common site within the [^{3}H]-nimodipine labelled calcium channel.

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