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The Ca^{2+}-antagonist and binding properties of the phenylalkylamine, anipamil

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1 Isolated, Langendorff-perfused rat hearts, isolated membranes, and pharmacological and receptor binding techniques were used to study the properties of the newly developed verapamil derivative, anipamil.

2 When added acutely to isolated, spontaneously beating or electrically paced hearts, anipamil (0.01–0.15 μM) exerted a dose-dependent negative inotropic effect which developed slowly and persisted after 60 min washout.

3 When added acutely (0.05–0.1 μM) to isolated hearts, or when given intravenously (2 mg kg^{-1} body weight 1 h before the animals were killed), anipamil displaced the dose-response curves for the positive inotropic effect of (0.10–3.0 mM) Ca^{2+} and (10–50 nM) Bay K 8644 to the right.

4 When added to freshly isolated cardiac membranes, 0.1 μM anipamil increased the dissociation constant (K_D) of the phenylalkylamine (−)-[^{3}H]-desmethoxyverapamil ((−)-[^{3}H]-D888) from 1.22 ± 0.2 to 2.91 ± 0.46 nM, without any significant change in density (B_{max}; control: 163 ± 17; anipamil: 117 ± 20 fmol mg^{-1} protein). Bound (−)-[^{3}H]-D888 was displaceable by (−)-D888 (K_i 1.7 ± 0.4 nM) > (−)-D600 (K_i 12 ± 0.5 nM) > verapamil (K_i 55 ± 11 nM) > (+)-D600 (K_i 108 ± 12.2) > anipamil (K_i 471 ± 52 nM).

5 In cardiac membranes isolated from rats pretreated with anipamil (2 mg kg^{-1} i.v.) 1 h before they were killed, the K_D of (−)-[^{3}H]-D888 binding was increased ($P < 0.05$) from 1.59 ± 0.18 to 3.28 ± 0.65 nM with no significant change in density, compared to the placebo-treated (control) rats.

6 These results establish that anipamil interacts in a competitive manner with the phenylalkylamine binding sites in cardiac membranes, and that it resembles other Ca^{2+} antagonists in displacing the dose-reponse curve for the positive inotropic effect of Ca^{2+} to the right. The results also show that although anipamil binds tightly to the cardiac membranes, it binds to the (−)-[^{3}H]-D888 recognition sites less potently than (−)-D888, (−)-D600 or verapamil.

Introduction

Anipamil (1,7-*bis*-(3-methoxyphenyl)-3-methylaza-7-cyano-nonadecane) is a newly developed verapamil derivative (Raschack, 1984) which protects the myocardium against damage caused by haemorrhagic shock (Farago *et al.*, 1984), ischaemia, and reperfusion (Gries & Raschack, 1984; Ferrari *et al.*, 1984; Curtis *et al.*, 1986). It also prevents platelet aggregation (Lehmann *et al.*, 1984; Osborne *et al.*, 1986). Papers describing experiments in which anipamil has been used (Bigoli *et al.*, 1984; Lehmann *et al.*, 1984; Osborne *et al.*, 1986; Curtis *et al.*, 1986) often refer to it as a 'calcium antagonist', simply because it was derived from verapamil. This need not necessarily follow. For example (−)-Bay K 8644 is a nifedipine derivative but whereas nifedipine is a potent Ca^{2+} antagonist (Vater *et al.*, 1972), (−)-Bay K 8644 is a potent agonist (Schramm *et al.*, 1983; Kokubun & Reuter, 1984). Likewise dichloroisoprenaline (DCI) was derived from isoprenaline, but whereas isoprenaline is a β-adrenoceptor agonist, DCI is a potent partial agonist.

The following experiments were undertaken to establish whether anipamil resembles other phenylalkylamine-based Ca^{2+} antagonists in terms of its effect on heart rate and force of contraction, on the dose-response curves for the positive inotropic effect of Ca^{2+} and the Ca^{2+} agonist Bay K 8644, and its interaction with membrane-located phenylalkylamine binding sites.

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Methods

Hearts from adult (250–300 g) male Sprague Dawley rats were used for these experiments. The hearts were isolated under ether anaesthesia and placed in ice-cold Krebs-Henseleit buffer until contractions ceased. They were then either perfused, or homogenized to provide membranes for binding studies.

The effect of anipamil on peak developed tension and heart rate, and on the positive inotropic effect of Ca^{2+} and Bay K 8644

The effect of anipamil on peak developed tension and heart rate To establish whether anipamil resembles verapamil (Nayler *et al.*, 1986; Nayler & Szeto, 1972), in having a direct negative inotropic effect on the heart, anipamil (0.01–0.15 μM) was added directly to isolated Langendorff-perfused rat hearts. Perfusion was with Krebs-Henseleit (K-H) buffer containing (in mM): NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11.0 and $CaCl_2$ 1.3. The buffer was gassed with 95% O_2 + 5% CO_2 (pH 7.4) and delivered at a constant flow rate of 10 ml min^{-1} g^{-1} heart weight. The hearts were either allowed to beat spontaneously, or were paced to beat at a constant rate of 180 beats min^{-1}, with square wave pulses of 10 ms duration delivered from a Tektronix square wave generator (Tektronix Inc., Portland, Oregon, USA). When electrically paced hearts were used the sinus node was crushed, and pulses from the stimulator were applied through two leads, one attached to the metal inflow cannula and the other to the ventricular apex. The output from the stimulator was adjusted to provide stimuli which were just suprathreshold. A rate of 180 beats min^{-1} was used because it falls within the range (182 ± 12 beats min^{-1}, Table 1) exhibited by hearts excised from anipamil-pretreated rats (Table 1). Hearts from the placebo-treated and untreated rats had a higher ($P < 0.05$) heart rate (262 ± 12, and 268 ± 15 beats min^{-1} respectively).

Peak developed tension was detected by means of a Narco F-60 Biosystem myograph attached to the left ventricular apex (Nayler & Scott, 1982). The output from the strain gauge was displayed on a Devices Physiograph recorder (Narco Biosystems, Houston, Texas). A 'resting' tension (2–3 g) was applied until the developed tension (difference between systolic and diastolic) was maximal. This

Table 1 Peak developed tension and heart rate of hearts isolated from untreated (control), placebo-treated (ethanol) and anipamil-pretreated rats

Minutes of perfusion	*Peak developed tension* (g)	*Heart rate* (beats min^{-1})
A. *Spontaneously beating preparations*		
(i) Control (untreated)		
130	9.6 ± 0.8	268 ± 5
390	9.2 ± 1.1	271 ± 12
(ii) Placebo (ethanol)		
30	9.9 ± 1.6	262 ± 12
390	9.4 ± 0.5	266 ± 9
(iii) Anipamil (2 mg kg^{-1}, i.v.)		
30	6.6 ± 0.4*	182 ± 12*
390	6.2 ± 0.7*	212 ± 14*
B. *Electrically paced hearts (180 beats min^{-1})*		
(i) Control (untreated)		
30	10.1 ± 0.6	
390	9.8 ± 1.0	
(ii) Placebo (ethanol)		
30	8.2 ± 0.6	
390	8.4 ± 0.4	
(iii) Anipamil (2 mg kg^{-1} i.v.)		
30	5.3 ± 0.5*	
390	5.6 ± 0.8*	

Each result is mean ± s.e. of mean of 6 experiments.

The hearts were perfused for 30 min to allow for equilibration, and then for another 3 h. Perfusion buffer was K-H solution containing 1.3 mM Ca^{2+}.

* Significant change (at $P < 0.01$ level) caused by anipamil pretreatment when compared to placebo (ethanol).

'resting' tension was maintained throughout each experiment. Heart rate was obtained from the mechanogram.

Each heart was perfused with K-H buffer for 30 min before the dose-response curve for the inotropic effect of anipamil was established. Anipamil was added cumulatively in aliquots of less than 20 $\mu l\,l^{-1}$ perfusion buffer. The same protocol was followed for the vehicle, which was ethanol. Only two concentrations of anipamil (separated by 60 min) were added to each heart. Pilot experiments showed that the maximum inotropic and chronotropic response developed within 60 min.

The effect of acutely administered anipamil on the positive inotropic effect of Ca^{2+} and Bay K 8644 In these experiments isolated, electrically-paced hearts were used, but the Ca^{2+} content of the perfusion buffer was reduced from 1.3 to 0.65 mM to ensure that the hearts were not contracting maximally. After 30 min perfusion dose-response curves were established for either Ca^{2+} (0.10–3.0 mM) or Bay K 8644 (10–50 nM). Cumulative doses of either Ca^{2+} or Bay K 8644 were not used. Instead the required concentration of either Ca^{2+} or Bay K 8644 was added as a bolus (0.1 ml) by way of a side arm. An interval of 15 min separated each dose, and during that time perfusion was continued with perfusion buffer containing 0.65 mM Ca^{2+}.

After obtaining control dose-response curves for Ca^{2+} and Bay K 8644 the effect of anipamil (0.1 μM) on these curves was established by adding anipamil directly to the perfusion buffer 60 min before the bolus additions of Ca^{2+} and Bay K 8644 were repeated as described above. Anipamil was present in the perfusion buffer throughout and care was taken to prevent Bay K 8644 from being exposed to light. Pilot experiments showed that up to 60 min was required for the negative inotropic effect of anipamil to reach asymptote.

The effect of anipamil pretreatment on the inotropic activity of Ca^{2+} and Bay K 8644 To establish whether pretreatment with anipamil antagonizes the positive inotropic effect of either Ca^{2+} or Bay K 8644, hearts were isolated from rats 1 h after they had been injected i.v. with either 0.1 ml ethanol or 0.1 ml ethanol containing 2.0 mg kg^{-1} anipamil. This amount of anipamil was calculated to provide a peak plasma concentration of approximately 40 μM and was used because pilot experiments established that it caused a rightward shift in the dose-response curve for the positive inotropic effect of Ca^{2+}.

The hearts were perfused as described above, and dose-response curves for either Ca^{2+} or Bay K 8644 established. Aliquots of Ca^{2+} or Bay K 8644 were added by way of a side arm, as described above, with 15 min between each addition. The Ca^{2+} content of the perfusion buffer was 0.65 mM and anipamil was not added. Only 3 doses of either Ca^{2+} or Bay K 8644 were added to each heart, to avoid any problem of tachyphylaxis. The hearts were paced to beat at 180 beats min^{-1}, pilot studies having established that hearts from the anipamil pretreated rats could be consistently stimulated to beat at this rate. In the placebo-treated group the sinus node was crushed, and the hearts were paced at 180 beats min^{-1}.

The interaction of anipamil with phenylalkylamine binding sites

Membrane isolation Cardiac membranes were isolated as described by Glossmann & Ferry (1985). The ventricles were minced into 1 mm cubes, and homogenized in ice-cold 20 mM $NaHCO_3$, 0.1 mM phenylmethylsulphonylflouride (PMSF), with a wet weight to volume ratio of 1:3, and using one 10 s burst of an Ultra Turrax (Kika Werk) homogenizer operating at $\frac{3}{4}$ maximum speed. The resultant homogenate was diluted 1:7 with buffer containing 20 mM $NaHCO_3$ and 0.1 mM PMSF, and then centrifuged at 1500 *g* for 15 min at 4°C in a Sorvall RC 2-B centrifuge, with a fixed angle SS 34 rotor. The supernatant was centrifuged at 48 000 *g* for 15 min at 4°C. The pellet from this spin was suspended in 40 ml of ice-cold buffer containing 50 mM Tris HCl and 0.1 mM PMSF, pH 7.4, and recentrifuged at 48 000 *g* for 15 min at 4°C. This procedure was repeated once. The resultant pellet, which would contain membranes from myocytes, smooth muscle cells and endothelial cells, was suspended in 50 mM Tris HCl, containing 0.1 mM PMSF (pH 7.4), to provide a final concentration of 0.8 to 1.2 mg protein ml^{-1}. Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

(−)-[^{3}H]-desmethoxyverapamil ((−)-[^{3}H]-D888) binding (−)-[^{3}H]-D888 binding was monitored as described by Glossmann & Ferry (1985). (−)-[^{3}H]-D888 was used as the ligand instead of [^{3}H]-verapamil (Dillon & Nayler, 1987) because it has a higher affinity for the phenylalkylamine binding sites and less non specific binding to membranes and filters, relative to [^{3}H]-verapamil (Goll *et al.*, 1984; Ferry *et al.*, 1984). Binding was performed in triplicate at 25°C for 60 min, using a protein concentration of 0.16–0.24 mg ml^{-1} in a final volume of 250 μl. The reaction mixture contains 50 mM Tris, 0.1 mM PMSF, pH 7.4. For saturation binding, 0.03–10 nM (−)-[^{3}H]-D888 was used. Non-specific binding was defined by adding 1 μM (−)-gallopamil ((−)-D600). After 60 min incubation, bound and free (−)-[^{3}H]-D888 were separated after dilution with

3.5 ml ice-cold 50 mM Tris buffer containing 6.6% polyethyleneglycol 6000, pH 7.4. Separation was achieved by rapid vacuum filtration (Dillon & Nayler, 1987), across Whatman GF/C filters, followed by two additional washes with the above buffer. The radioactivity of the filters was counted (40% efficiency) in Filter Count Scintillant (Packard, Illinois, U.S.A.), with a Packard Tricarb Spectrometer. Binding selectivity was characterized by use of (−)-D888 (1 pM–1 μM), (−)-D600 (0.1 nM–10 μM), (+)-D600 (0.1 nM–10 μM), (−)-verapamil (0.1 nM–10 μM) and (−)-Bay K 8644 (10 pM–10 μM) to displace bound (−)-[^{3}H]-D888.

Effect of anipamil on (−)-[^{3}H]-D888 binding (in vitro) Two strategies were used to monitor the effect of anipamil on (−)-[^{3}H]-D888 binding. In one series a single dose of anipamil (0.1 μM) was added to the incubation buffer and saturation (−)-[^{3}H]-D888 binding isotherms established. This enabled us to ascertain whether anipamil was a competitive, partially competitive or non-competitive ligand for the phenlyalkylamine (−)-[^{3}H]-D888 binding site. The second strategy involved construction of displacement curves to gain information relating to the apparent affinity and selectivity of anipamil for the (−)-[^{3}H]-D888 binding sites.

Anipamil was dissolved either in absolute ethanol or DMSO, to provide a stock solution of 10^{-3} M. Subsequent dilutions were prepared with incubation buffer. Pilot studies were also undertaken in which methanol/-cremophor EL (100:1) was used to produce a stock solution of 10^{-3} M, followed by dilution with water/cremophor EL (100:1).

(−)-[^{3}H]-D888 binding after intravenous pretreatment with either anipamil or verapamil Rats were injected i.v. with a single dose of either 2.0 mg kg^{-1} anipamil dissolved in 0.1 ml ethanol, or 0.1 ml of ethanol. One hour later the hearts were excised, the cardiac membranes isolated, and (−)-[^{3}H]-D888 binding monitored as already described. Ethanol-injected rats provided controls for this study.

To compare the effect of anipamil pretreatment on D888 binding with the effect of pretreatment with another phenylalkylamine, a single dose of verapamil (1 mg kg^{-1}) dissolved in 0.1 ml ethanol was administered i.v. to another group of rats 1 h before they were killed. This dose (1 mg kg^{-1}) was the maximum tolerated by the rats, when given as a bolus.

Reagents

(−)-[^{3}H]-D888, as (−)-[N-methyl-^{3}H]-desmethoxyverapamil (specific activity, 60 Ci mmol^{-1}) was obtained from Amersham Laboratories (Buckinghamshire, England). (−)-Bay K 8644 methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was obtained as a gift from Bayer AG, Wupperthal, Germany, (−)-D888-HCl, (+)-D600 HCl, (−)-verapamil HCl, (−)-D600-HCl and anipamil-HCl-hydrate were gifts from Knoll AG, Ludwigshafen, Germany. Anipamil was dissolved in ethanol. Polyethyleneglycol (PEG) 6000 was obtained from BDH Chemicals, Australia. Cremophor EL and all other reagents were from Sigma Chemical Company, St. Louis, U.S.A.

Analysis of results

Initial estimates of equilibrium binding parameters (K_D and B_{max}) were obtained by Scatchard, Hill and Hofstee analysis, using the 'EBDA' programme (McPherson, 1983).

A file was then produced and the data analysed with the aid of a weighted, non-linear, least square computer curve fitting programme (Munson & Rodbard, 1980). The binding data were subjected to Student's t test at the $P < 0.05$ level of significance.

Results

Control studies

Pilot studies showed that when hearts from control (untreated) and placebo (ethanol)-treated rats were perfused with Krebs-Henseleit buffer for 360 min, after the initial 30 min equilibration peak developed tension remained constant. This applied (Table 1) to spontaneously beating and to the electrically paced hearts. In the spontaneously beating hearts, the heart rate also remained constant (Table 1). These results show, therefore, that the preparations are stable for 390 min.

Hearts from the anipamil-pretreated rats also remained stable when perfused for 3 h after the initial 30 min equilibration. Peak developed tension was steady (Table 1) irrespective of whether the hearts were electrically paced or allowed to beat spontaneously. The spontaneously beating hearts from the anipamil pretreated rats exhibited a small but non-significant increase in heart rate over the 3 h period (from 182 ± 12 to 212 ± 14 beats min^{-1}, Table 1).

Effect of anipamil pretreatment on peak developed tension and heart rate

Table 1 shows that anipamil pretreatment caused a significant ($P < 0.01$) reduction in peak developed tension in both spontaneously beating and electrically-paced hearts. This effect persisted

throughout 390 min perfusion with Krebs-Henseleit buffer. In addition, and although no anipamil was added to the perfusion buffer, the spontaneous rate of hearts isolated from the anipamil-pretreated rats was slow (182 ± 12 beats min^{-1}) relative to that obtained for hearts from placebo-treated (262 ± 12 beats min^{-1}) or untreated (268 ± 15 beats min^{-1}) rats (Table 1).

These results show that the isolated perfused rat heart is a stable preparation, and that pretreatment with anipamil (2 mg kg^{-1} body wt, i.v.) 1 h before excising the hearts results in a slowed spontaneous heart rate and a decline in peak developed tension (Table 1).

The effect of anipamil on peak developed tension and the positive inotropic effect of either $CaCl_2$ of Bay K 8644 on isolated electrically paced hearts

Figure 1 shows that when added to isolated, electrically-paced hearts anipamil (0.01–0.15 μM) evoked a dose-dependent negative inotropic response. This effect developed relatively slowly. For example, the response to 10 nM anipamil took 65 ± 10 min (mean $\pm$ s.e. mean, $n = 3$) to reach asymptote. When 0.1 μM anipamil was added, only 38 ± 8 min (mean $\pm$ s.e. mean, $n = 3$) was needed.

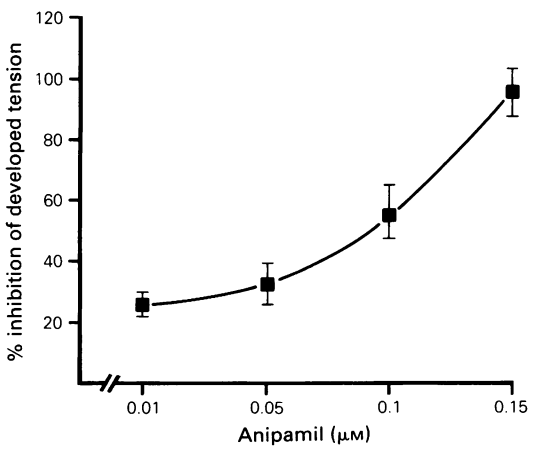

Figure 1 Dose-response curve for the negative inotropic effect of 0.01–0.15 μM anipamil on isolated Langendorff perfused rat hearts stimulated to beat 180 min^{-1}. The perfusion buffer was a modified K-H solution containing 1.3 mM Ca^{2+}. Percentage inhibition of peak developed tension was calculated relative to the peak tension developed before any anipamil was added. Only 2 concentrations of anipamil (■) were added to each heart. Each point is mean of 4 separate experiments with s.e. mean shown by vertical lines. After equilibration and before any anipamil was added the hearts developed 9.6 ± 0.3 g tension.

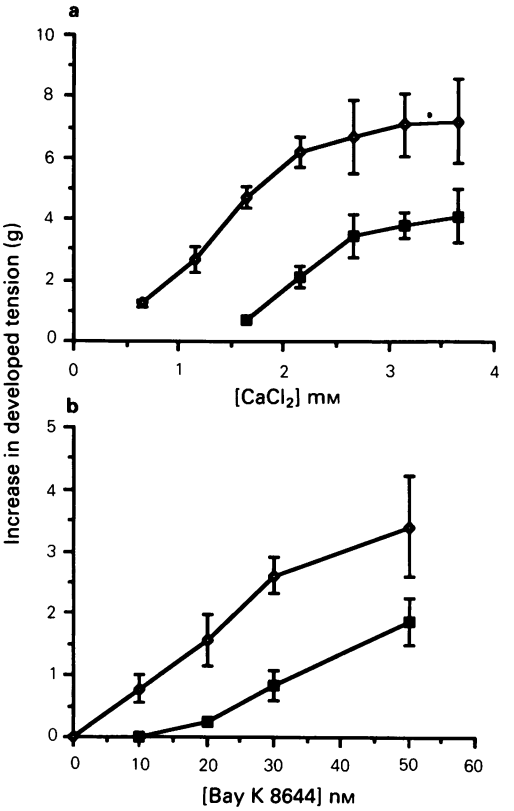

Figure 2 Dose-response curves for the positive inotropic effect of (a) 0.1–3.0 mM Ca^{2+}, and (b) 10–50 nM Bay K 8644 when added before (◇) and 60 min after (■) adding 0.1 μM anipamil to isolated, electrically paced (180 min^{-1}) hearts perfused with K-H buffer containing 0.65 mM Ca^{2+}. Each point is mean of 4 experiments; vertical lines show s.e. mean. Ca^{2+} and Bay K 8644 were added as individual doses, and no more than 3 doses were added to any one heart. Increase in developed tension was calculated relative to the peak tension developed immediately before adding the additional Ca^{2+} (as $CaCl_2$) or Bay K 8644. The Ca^{2+} concentration in (a) refers to the final Ca^{2+} concentration present when the test dose was added to the buffer which already contained 0.65 mM Ca^{2+}. After equilibration and before any extra Ca^{2+} or Bay K 8644 was added the control hearts developed 5.9 ± 0.8 g tension, whereas hearts from the anipamil-pretreated rats developed 3.6 ± 0.5 g.

Figure 2a and b shows the dose-response curves obtained for the positive inotropic effect of Ca^{2+} (0.10–3.0 mM Ca^{2+}) and Bay K 8644 (10–50 nM) (Figure 2a and b respectively) before and 60 min after adding 0.1 μM anipamil. In both cases anipamil displaced the dose-response curves to the right.

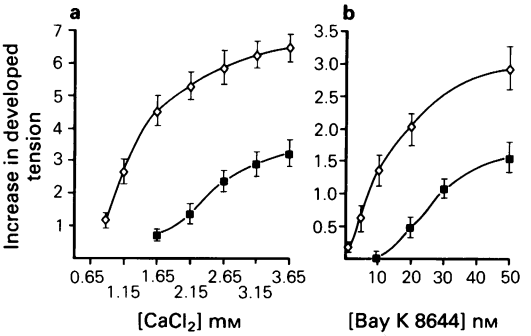

Figure 3 Dose-response curves for the positive inotropic effect of (a) 0.1–3.0 mM Ca^{2+} and (b) 10–50 nM Bay K 8644 added to hearts isolated from anipamil (2 mg kg^{-1}) pretreated (■) or placebo (ethanol) pretreated (◇) rats. The hearts were electrically paced (180 beats min^{-1}) and were perfused with K-H buffer (see text) containing 0.65 mM Ca^{2+}. Each point is the mean of 4 experiments; vertical lines show s.e. mean. Ca^{2+} and Bay K 8644 were added as individual non-cumulative doses, with no more than 3 doses being added to each heart. The Ca^{2+} concentrations shown in (a) refer to the final Ca^{2+} concentration present in the perfusion buffer (0.65 mM Ca^{2+} plus the test dose). The increase in peak developed tension was calculated relative to the peak tension developed immediately before adding extra Ca^{2+} (as $CaCl_2$) or Bay K 8644. Hearts from the anipamil-pretreated and placebo (ethanol) pretreated rats developed 4.0 ± 0.4 g and 5.4 ± 0.3 g tension respectively during perfusion with 0.65 mM Ca^{2+} before the additional Ca^{2+} or any Bay K 8644 was added.

Addition of ethanol in an amount equivalent to that used as the vehicle for anipamil (<0.1 ml) had no effect on either peak developed tension or the dose-response curves for the inotropic effect of Ca^{2+} or Bay K 8644 over the dose-range used here.

The effect of anipamil i.v. pretreatment on the positive inotropic effect of Ca^{2+} *and Bay K 8644*

Figure 3 shows that pretreatment of rats with anipamil (2 mg kg^{-1} body weight, i.v.) 1 h before the hearts were excised resulted in the dose-response curves for the positive inotropic effect of $CaCl_2$ and Bay K 8644 being displaced to the right, relative to the results obtained for hearts from ethanol (0.1 ml) injected rats. The results shown in Figure 3 were obtained from electrically-paced hearts perfused with K-H buffer containing 0.65 mM Ca^{2+}. Similar results (not shown) were obtained when spontaneously beating preparations were used.

The differences in peak developed tension and heart rate (Table 1) and the rightward shift in the dose-response curves for Ca^{2+} and Bay K 8644 (Figure 3) indicate that the effect of anipamil pretreatment persists after the hearts have been isolated. The slowed heart rate and diminished peak developed tension persisted for up to 5 h. Dose-response curves were constructed within this time limit.

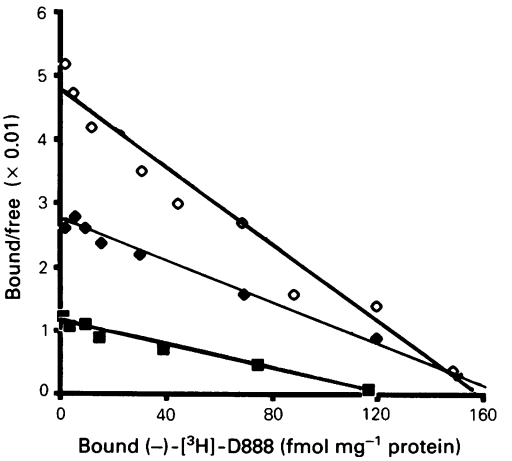

Figure 4 Representative Scatchard plot of (−)-[^{3}H]-D888 binding to rat cardiac membrane fragments (◆), and to fragments incubated with ethanol (◇), and with ethanol plus (0.1 μM) anipamil (■). The binding parameters are given in Table 1. Ordinate scale: bound/free (× 0.01); abscissa scale: bound (−)-[^{3}H]-D888 (fmol mg^{-1} protein). Similar estimates were obtained from 6 separate experiments; triplicate estimates were used for each point.

The interaction of anipamil with the phenylalkylamine binding sites

(−)-[^{3}H]-D888 binding and the effect of anipamil Membrane fragments obtained from freshly excised hearts of untreated rats contained a single population of high affinity (−)-[^{3}H]-D888 binding sites (Figure 4). Binding was saturable, reversible and selectively displaceable. Scatchard analysis revealed that these sites had an affinity (K_D) of 2.07 ± 0.25 nM and a density (B_{max}) of 172 ± 15.3 fmol mg^{-1} protein (Table 2). Ethanol (0.01% v/v) decreased their K_D (1.22 ± 0.21 nM) but the B_{max} (153 ± 17.3 fmol mg^{-1} protein) (Table 2) was unchanged. Addition of 0.1 μM anipamil (in ethanol) reduced ($P < 0.05$) the affinity of these binding sites without changing their density (Table 2). In each case the Hill coefficients centred around unity, indicating a single population of (−)-[^{3}H]-D888 binding sites. Figure 5 shows that anipamil displaced bound (−)-[^{3}H]-D888 less potently (K_i 471 ± 52 nM) than (−)-D888 (K_i 1.7 ± 0.4 nM), (−)-

Table 2 The acute effect of anipamil (0.1 μM) on (−)-[^{3}H]-D888 binding in rat cardiac membrane fragments

	K_D (nM)	B_{max} (fmol mg^{-1} protein)	*Hill coefficient*
Control	2.07 ± 0.25	172 ± 15.3	1.043 ± 0.059
Ethanol (0.01% v/v)	1.22† ± 0.21	153 ± 17.3	1.028 ± 0.094
DMSO (0.01% v/v)	2.10 ± 0.15	169 ± 17.1	1.013 ± 0.008
Anipamil in ethanol (0.1 μM)	2.91* ± 0.46	117 ± 20	0.997 ± 0.023
Anipamil in DMSO (0.1 μM)	4.2** ± 0.54	119** ± 18.1	0.989 ± 0.012

† Significant change ($P < 0.05$) caused by ethanol with respect to control.
* Significant change ($P < 0.05$) caused by the acute addition of anipamil (prepared in ethanol) with respect to the vehicle (ethanol) control.
** Significant change ($P < 0.05$) caused by anipamil prepared in dimethylsulphoxide (DMSO) with respect to the vehicle (DMSO) control.
Each result is mean ± s.e. mean of 6 separate experiments.

D600 (K_i 12 ± 0.5 nM), verapamil (K_i 55 ± 11 nM), or (+)-D600 (K_i 108 ± 12.2 nM). (−)-Bay K 8644 (IC_{50} 2.0 ± 0.24 nM) was used to check selectivity.

To determine whether the change in the dissociation constant of (−)-[^{3}H]-D888 binding caused by anipamil (prepared in ethanol) was due to the presence of ethanol, dimethoxysulphoxide (DMSO) instead of ethanol was used as the vehicle for anipamil. Under these conditions anipamil increased the dissociation constant, from the control value of 2.10 ± 0.25 to 4.2 ± 0.54 nM. This change resembles that obtained when ethanol was used as the placebo (Table 1). The B_{max} was also reduced, from 169 ± 17.3 to 119 ± 18.1 fmol mg protein^{-1} (Table 2).

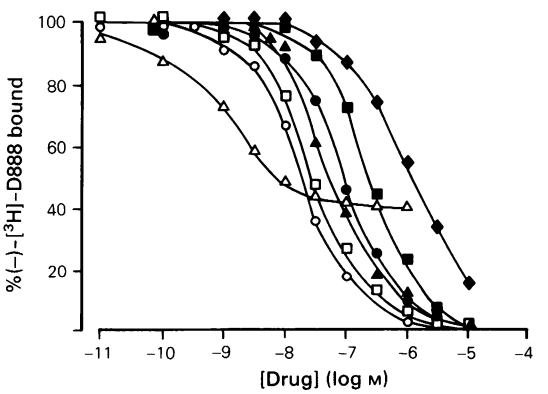

Figure 5 Displacement by (−)-D888 (○), (−)-D600 (□), (+)-D600 (■), (−)-verapamil (●), (−)-Bay K 8644 (△) and anipamil in ethanol (◆) or cremophor EL (▲) of (−)-[^{3}H]-D888 bound to cardiac membranes freshly isolated from untreated rats. The displacement parameters are given in the text. Ordinate scale: % bound; Abscissa scale: [Drug] (log M). Similar estimates were obtained from 4 separate experiments; triplicate estimates were used for each point.

(−)-[^{3}H]-D888 binding after i.v. pretreatment with either anipamil or verapamil Cardiac membranes obtained from rats which had been injected i.v. with 0.1 ml ethanol (the diluent for anipamil) 1 h before they were killed contained a single population of (−)-[^{3}H]-D888 binding sites. These sites had a K_D of 1.59 ± 0.18 nM and a B_{max} of 191 ± 19 fmol mg^{-1} protein (Table 3), values which are similar to those obtained for membranes which had been isolated from untreated animals but incubated in the presence of ethanol (Table 2).

Membranes prepared from rats killed 1 h after they were injected with 2 mg kg^{-1} i.v. anipamil also contained a single population of (−)-[^{3}H]-D888 binding sites. These sites had a K_D of 3.28 ± 0.65 nM, which is higher ($P < 0.05$) than that obtained for membranes from ethanol-pretreated rats (K_D 1.59 ± 0.18 nM). Anipamil pretreatment had no effect on the density of these sites (B_{max} 186 ± 17 fmol mg^{-1} protein for the anipamil pretreated group, 191 ± 19 fmol mg^{-1} protein (Table 3) for the ethanol controls).

Verapamil (1 mg kg^{-1}, i.v.) pretreatment (for 60 min) had no effect on the affinity (K_D 2.05 ± 0.21 nM) or density (B_{max}, 225 ± 15.6 fmol mg^{-1} protein) of the (−)-[^{3}H]-D888 binding sites, compared to the ethanol controls (Table 3).

Table 3 Effect of either anipamil or verapamil pretreatment on (−)-[^{3}H]-D888 binding in rat cardiac membrane fragments

Intravenous injection of	K_D (nM)	B_{max} (fmol mg^{-1} protein)	*Hill coefficient*
Ethanol (0.1 ml) ($n = 12$)	1.59 ± 0.18	191 ± 19	1.025 ± 0.081
Anipamil in ethanol (2 mg kg^{-1}) ($n = 6$)	3.28* ± 0.65	186 ± 17	1.013 ± 0.089
Verapamil in ethanol (1 mg kg^{-1}) ($n = 6$)	2.05 ± 0.21 NS	225 ± 15	0.989 ± 0.026

* Significant change ($P < 0.05$) caused by pretreatment with anipamil (2 mg kg^{-1}) relative to control (ethanol). NS–not significant.
Anipamil, verapamil and ethanol were injected i.v. 1 h before the rats were killed.
Each result is mean ± s.e. mean of n experiments.

Discussion

These results show that the addition of anipamil to isolated spontaneously beating or electrically paced rat hearts provokes a dose-dependent negative inotropic effect which is slow in onset. In spontaneously beating preparations this negative inotropism is accompanied by a slowed heart rate. Comparable changes in heart rate and force of contraction were evident in hearts excised from rats which had been pretreated with anipamil, although here no attempt was made to establish a dose-response relationship nor was any attempt made to determine whether a metabolite of anipamil contributed to the response, because as yet little is known about the chemistry, pharmacological activity, pharmacokinetics or binding profiles of the anipamil metabolites.

The changes in heart rate and force of contraction caused by anipamil pretreatment appear to be long lasting in the sense that the effect persisted for several hours after excising the hearts. Since rat hearts have a negative 'staircase', the cardiodepressant activity of anipamil cannot be accounted for in terms of its heart rate slowing activity. In any case, the negative inotropic effect of anipamil was evident in electrically paced preparations. Whether this heart rate slowing effect is due to slowed pacemaker activity, slowed impulse conduction or a combination of both requires further electrophysiological studies. The possibility of the slowed heart rate and decreased force of contraction displayed by hearts excised from anipamil pretreated rats being due to a metabolite that remained bound to the heart will also need further study. However, this was not the main purpose of this study; instead we were interested in describing the binding properties of anipamil and determining whether it exhibited any of the characteristics required for classification as a Ca^{2+} antagonist.

Negative inotropism and chronotropism alone certainly do not justify classifying a drug as a calcium antagonist. The present results, however, provide two reasons for placing anipamil in this category. Firstly, the drug displaced the dose-response curves for the positive inotropic effect of Ca^{2+}, and of the Ca^{2+} agonist Bay K 8644, to the right. Secondly, it interacted competitively with phenalykylamine binding sites, a site through which other phenylalkylamine-based Ca^{2+} antagonists mediate their activity (Ruth *et al.*, 1985; Dillon & Nayler, 1987; Glossmann & Ferry, 1985). Since this competitive interaction was observed when anipamil was added either to isolated membranes, or given intravenously before isolating the hearts and harvesting the membranes, it is unlikely that the interaction is due solely to an effect of a metabolite, because if this were the case, no such effect should have been obtained following the direct addition of the drug to the membranes. The membranes used for these binding studies were heterogeneous, being derived from a homogenate which would necessarily contain cardiac myocytes, vascular smooth muscle cells, and endothelial cells. For this reason no attempt is made here to extrapolate dose-response data obtained from intact hearts to data obtained from membrane fragments.

Although anipamil interacted with the (−)-[^{3}H]-D888 binding sites in a competitive manner it appeared to be less potent than either (−)-D888, (−)-D600 or (−)-verapamil in displacing (−)-[^{3}H]-D888. There are several reasons why this result might have been obtained. The binding of anipamil may not be limited to the D888 recognition fraction of the phenylalkylamine binding site. It may interact

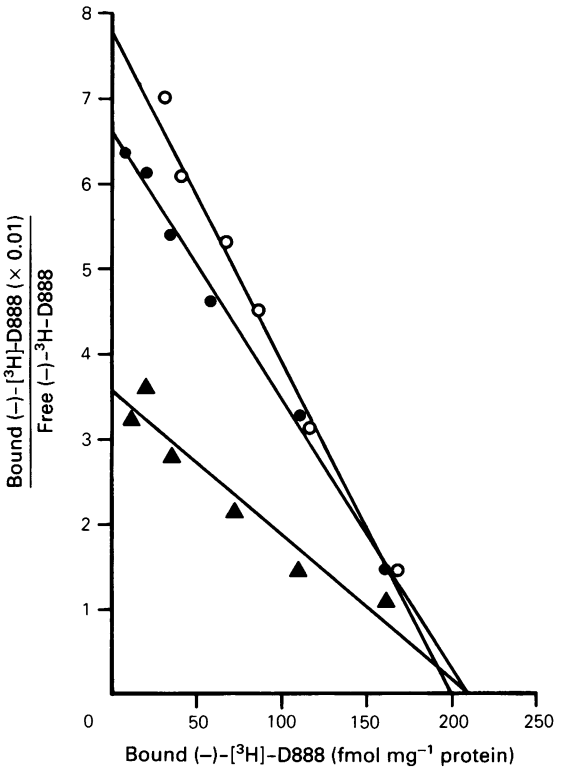

Figure 6 Representative Scatchard plot of (−)-[^{3}H]-D888 binding to cardiac membranes isolated from rats which had been injected i.v. with (0.1 ml) ethanol (○), verapamil (1 mg kg^{-1}) (●) or anipamil (2 mg kg^{-1}) (▲) in 0.1 ml ethanol 1 h before they were killed. The binding parameters are given in Table 3. Ordinate scale: bound/free (× 0.01); Abscissa scale: bound (−)-[^{3}H]-D888 (fmol mg^{-1} protein). Similar estimates were obtained from 6 separate experiments; triplicate estimates were used for each point.

with adjacent site(s). Alternatively there may be a solubility problem. In pilot studies this possibility was investigated by using an alternative vehicle, methanol/cremophor EL (ratio 100:1) with further dilutions with water/cremophor EL (ratio 100:1). This regime produced an apparent increase in the potency of anipamil as a displacer of bound D888, (K_i of 36 ± 10 nM). However, at the same time the total D888 binding capacity of the membrane was reduced by 40%, even in the absence of anipamil. We have interpreted this to mean that cremaphor EL, whilst being a good solvent for anipamil, interacts directly with the D888 binding sites in such a way as to make it unsuitable for use in these binding studies. As far as anipamil is concerned there may be the added problem in that, like its parent compound, it may adhere to glass and plastic surfaces, thereby reducing its total free concentration. Until radioactively labelled anipamil with high specific activity becomes available this possibility cannot be excluded.

One of the interesting points that emerged during this study was the fact that anipamil which had been given intravenously, remained bound to cardiac membrane fragments throughout homogenization and a relatively long isolation procedure which included two washes. Evidence of this persistent binding is provided by the change in K_D. When the same protocol was followed for verapamil instead of anipamil, the affinity of the D888 binding sites in the isolated membranes was unchanged (Table 3). Presumably therefore, verapamil, but not anipamil, was lost from the membrane fragments during the process of homogenization and purification. When added directly to freshly harvested membranes, however, verapamil displaced (−)-[^{3}H]-D888, confirming its ability to interact with phenylalkylamine recognition sites. Although our results show that the binding of anipamil to the phenylalkylamine recognition sites outlives that of verapamil, this may only account in part for its persistent action *in vivo*. If this had been the only explanation, we would have expected the D888 displacement studies to reveal a K_i for anipamil less than that of verapamil. Even when methanol/cremophor was used as the diluent for anipamil this did not occur. Possibly, as Curtis *et al.*, (1986) have suggested, the long action of anipamil is due to its molecular structure, since its long side chain would probably increase its lipophilicity and hence retention in the lipid bilayer.

A long-lasting association of anipamil with its receptors supports the findings of Raschack (1984), and Gries & Raschack (1984), who reported that anipamil protects the myocardium against the consequences of oxygen deprivation for a long time, relative to the protection provided by nifedipine, verapamil or tiapamil. Evidence of a long lasting effect of anipamil was obtained in the present study, because even when perfused with anipamil-free K-H buffer, hearts from anipamil-pretreated rats exhibited a negative inotropic effect and a slowed heart rate which persisted for several hours.

In some of the present experiments ethanol was used as the vehicle for anipamil. Posner *et al.* (1986) have already shown that ethanol potentiates the negative chronotropic effect of verapamil and because of this they concluded that ethanol and verapamil act synergistically at the level of the slow Ca^{2+} channels. The present study shows that ethanol actually interacts with the phenylalkylamine binding sites, increasing their affinity without changing their density. This could account for the synergism described by Posner *et al.* (1986). Other

circumstances in which the affinity of the phenylalkylamine binding sites increases is that of ischaemia (Dillon & Nayler, 1987) and under these conditions the myocardium becomes hypersensitive to the negative inotropic activity of verapamil (Smith *et al.*, 1976). Possibly, therefore, it is the affinity of these binding sites, rather than their density, which is primarily responsible for determining tissue sensitivity.

In conclusion, the present results show that anipamil interacts competitively with the phenylalkylamine binding sites in rat cardiac membranes, an effect which, in contrast to verapamil, survives membrane isolation. Our results also show that anipamil exerts a dose-dependent negative inotropic and chronotropic effect on the heart and that it displaces the dose-response curves for the positive inotropic effect of Ca^{2+} and Bay K 8644 to the right. In these respects anipamil resembles other Ca^{2+} antagonists but electrophysiological studies are now required to endorse the claim that anipamil is a Ca^{2+} antagonist.

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