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Tissue response selectivity of calcium antagonists is not due to heterogeneity of [^{3}H]-nitrendipine binding sites

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1 [^{3}H]-nitrendipine binding data and isolated tissue response for five calcium antagonists were evaluated in rabbit myocardium and aorta.

2 The [^{3}H]-nitrendipine binding site was qualitatively identical in myocardium and aorta, as the [^{3}H]-nitrendipine K_D, K_Is for nicardipine and nifedipine and interactions with verapamil, D600 and diltiazem were not different in aortic and cardiac membranes prepared by similar means.

3 In contrast, the inhibition of the Ca^{2+}-induced contractile response in right ventricular myocardium and aortic ring segments indicated a $>$ 10,000 fold selectivity of nicardipine for antagonism of vascular responses. This resulted in a different order of potency for calcium antagonist interaction with the [^{3}H]-nitrendipine binding site in cardiac membranes (nicardipine $>$ nifedipine $>$ D600 $>$ verapamil $>$ diltiazem) as compared to antagonism of myocardial tissue response (D600 $>$ verapamil $>$ nifedipine $>$ nicardipine$>$ diltiazem). In heart the difference between the potency of nicardipine in binding experiments and tissue response approached 4 orders of magnitude.

4 We conclude that tissue response selectivity of calcium antagonists is not explained by heterogeneity of [^{3}H]-nitrendipine binding sites.

Introduction

Recent evidence has convincingly demonstrated that the radiolabelled 1,4-dihydropyridine [^{3}H]-nitrendipine binds to discrete membrane binding sites that have many of the characteristics of pharmacological 'receptors'. That is, [^{3}H]-nitrendipine binding is of high affinity, obeys mass action laws, is stereospecific, and dissociation constants calculated from [^{3}H]-nitrendipine competition curves fall within the general *in vitro* potency range of 1,4-dihydropyridine 'calcium antagonists' (Bellemann *et al.*, 1981; 1982; Bolger *et al.*, 1982; Ehlert *et al.*, 1982a,b; Gould *et al* 1982; Murphy & Snyder, 1982; Williams & Tremble, 1982).

The concept of a specific receptor site to which calcium antagonists bind and exert their inhibitory effects on sarcolemmal calcium flux is attractive. Classical pharmacological studies have predicted the existence of such a site (Bristow & Green, 1977; Schramm *et al.*, 1983), and heterogeneity in such binding sites would explain the tissue selectivity possessed by some of these agents (Van Nueten & Wellens, 1979; Henry, 1980; Satoh *et al.*, 1980; Ginsburg *et al.*, 1981; Ogawa *et al.*, 1981; Quinn *et al.*, 1981; Walsh *et al.*, 1981). Two recent studies in vascular smooth muscle and myocardium of different species have in fact suggested differences in [^{3}H]-nitrendipine binding sites in these tissues, based on a $>$2 fold difference in K_D (Depover *et al.*, 1982; Williams & Tremble, 1982).

We therefore decided to characterize [^{3}H]-nitrendipine binding in myocardial and vascular smooth muscle membranes derived from the same species and compare binding data with antagonism of calcium-mediated tissue response, in order to test the hypothesis that response selectivity of calcium antagonists is related to their ability to recognize heterogenous binding sites. Included in the profile of calcium antagonists were 2 compounds that demonstrate selectivity for vascular smooth muscle response, diltiazem (Walsh *et al.*, 1981; Ginsburg *et al.*, 1981) and nicardipine (Satoh *et al.*, 1981). The data indicate that [^{3}H]-nitrendipine binding sites in rabbit myocardium are identical to those in aorta and that the selectivity of nicardipine and diltiazem is not explained by differing characteristics of these binding sites. Furthermore, in myocardium the disparity between binding and physiological response data raises the question as to whether [^{3}H]-nitrendipine binding

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sites are the pharmacological site of action of 1,4-dihydropyridine calcium antagonists.

A preliminary account of this work has appeared in abstract form (Bristow *et al.*, 1982b).

Methods

Rabbit myocardial membranes suitable for receptor binding experiments were prepared by a modification of previously described methods (Bristow *et al.*, 1982a). Five g of right and left ventricular free wall (2.5 g of each myocardium pooled) were excised from 4 female New Zealand White rabbits (3.0–3.5 kg) randomly selected from those used for the harvest of aortic tissue (see below). The tissue was homogenized in 10 volumes of ice-cold 10 mM Tris, 1.0 mM EGTA buffer, pH 8.0 by means of 3 consecutive 5 s bursts on a Polytron (Brinkman Instruments, Westbury, New York) set at full speed. An equal volume of 1 M KCl was then added for extraction of contractile proteins and the suspension was continuously stirred for 15 min at 4°C. This homogenate was centrifuged at 50,000 g for 15 min, and the pellet resuspended in 250 mM sucrose, 5 mM Tris, 1.0 mM EGTA buffer, pH 7.8 by means of a 1 s burst on the Polytron. This suspension was then re-centrifuged and resuspended twice, using a buffer of 50 mM Tris, pH 7.8. The final resuspension was then given 10 passes on an automatic mortar and pestle and filtered through 4 layers of cheesecloth to give a suspension with a protein concentration of 1.5–3.5 mg ml^{-1}.

Rabbit aortic membranes were prepared as follows: 10–15 New Zealand White rabbits (3.0–3.5 kg) were given 1,000 units of heparin subcutaneously and killed by concussion 10–30 min later. The thoracic and abdominal aortae were dissected free and fastidiously cleaned of adherent fat and connective tissue while in oxygenated Tryode solution. Six-nine grams of pooled thoracic and abdominal aorta were then minced finely with scissors and homogenized in 10 mM Tris, 1 mM EGTA buffer, pH 8.0 by means of one 10 s burst on the Polytron at a setting of 7, followed by three 7 s bursts at full speed. Following the addition of an equal volume of 500 mM sucrose, 5 mM Tris, 1 mM EGTA buffer, pH 7.8, the homogenate was centrifuged at 250 *g* for 10 min, and the pellet discarded. An equal volume of 1 M KCl was then added to the supernatant, and the solution was continuously stirred at 4°C for 15 min. This suspension was then centrifuged at 50,000 *g* for 15 min, and the pellet then resuspended in 50 mM Tris pH 7.8 buffer, as above. Following a final centrifugation at 50,000 g and resuspension in 50 mM Tris buffer this final preparation was given 10 passes on an automatic mortar and pestle and filtered through 4 layers of cheesecloth to yield a protein concentration of 1.0–2.5 mg ml^{-1}.

[^{3}H]-nitrendipine bound to cardiac and aortic membranes was trapped by vacuum filtration (Bristow *et al.*, 1982a). For receptor density and [^{3}H]-nitrendipine affinity determinations, 5–8 increasing concentrations of [^{3}H]-nitrendipine were incubated with membranes in the presence and absence of 1 μM nicardipine. The buffer was 50 mM Tris, pH 7.45, with a final assay volume of 450 μl. Membrane protein concentration in cardiac membranes was 1.0–2.3 mg ml^{-1} and 0.7–1.7 mg ml^{-1} in aorta. For equilibrium studies the assay mixture was incubated for 30 min at 30°C, diluted with 5 ml of room temperature buffer, vacuum-filtered through 1 μm glass fibre filters (Gelman Corporation, Ann Arbor, Michigan) and washed with 20 ml of buffer at a flow rate of 2 ml s^{-1}. The filters were then dried and counted at 38–42% efficiency. Specific binding was calculated as that displaceable by 1 μM nicardipine. All assays were performed in triplicate.

Association kinetics of [^{3}H]-nitrendipine were determined by adding prepared cardiac membranes to an assay mixture containing 1.5 nM [^{3}H]-nitrendipine in the presence and absence of 1 μM nicardipine at a final assay volume of 450 μl. Dissociation kinetics were determined by incubating membranes in 1.5 nM [^{3}H]-nitrendipine for 30 min, then adding 1 μM nicardipine and interrupting the assay at varying times. The effects of calcium antagonists on competitive inhibitors of the binding of [^{3}H]-nitrendipine were determined by incubating 1.5 nM [^{3}H]-nitrendipine with membranes and varying concentrations of calcium antagonists for 30 min at 30°C in an assay volume of 450 μl. All binding experiments and preparation of light sensitive compounds were performed in the dark or in filtered light that eliminated wave lengths < 600 mμ.

The ability of calcium antagonists to inhibit physiological responses was assessed in isolated rabbit myocardial tissue and aortic ring segments by modification of previously described methods (Bristow & Green, 1977; Bristow *et al.*, 1982a; Ginsburg *et al.*, 1980a,b; 1983). Isolated left atria, bisected longitudinally into 2 halves (Bristow & Green, 1977) or 3 right ventricular wall muscle strips (0.5–0.7 × 6–7 mm) were placed in physiological salt solution (PSS, concentration mM: NaCl 118, KCl 4.0, $MgSO_4$ 1.2, $NaHCO_3$ 24.0, NaH_2PO_4 1.2, $CaCl_2$ 2.5; pH 7.40–7.45, 37°C). The ionized calcium (Ca^{2+}) concentration of this solution measured by ion-specific electrode (Applied Medical Technology, Palo Alto, California) was 1.25 mM. The muscle was then attached to a force-displacement transducer (Gould, UC-3).

Isometric contraction was generated by field

stimulation through platinum electrodes parallel to the long axis of the muscle (3.0 ms square wave pulse at 10% above threshold at 0.6 Hz). The initial length of each muscle strip was adjusted to obtain the maximal isometric tension. The actively developed tension was calculated as the difference between the peak tension during a contraction (total tension) and the resting tension (1–1.5 g). The tissue was equilibrated in PSS for 60 min, then incubated in the dark with the various calcium antagonists for 60 min. The PSS was then exchanged for a calcium-free solution (PSS without calcium but containing 0.5 mM EGTA). Cumulative concentration-response curves were generated by adding calcium chloride and ionized calcium was measured by an ion-specific electrode. For myocardial experiments, 3 right ventricular strips from each of 3 hearts were mounted in a multiple chamber bath. One strip from each heart was used to generate each concentration-response curve. Responses were recorded in the presence and absence of 2 concentrations of a calcium antagonist (nicardipine) or appropriate concentrations of two different calcium antagonists. Concentration-response curves were then constructed on a % of maximum basis relative to control. In four preliminary experiments the control EC_{50} displayed a coefficient of variation of 8.3%. Left atrial tissue was similarly studied, with one half of an atrium paired with one half of a second atrium for analysis of concentration-response curves.

Rabbit thoracic aorta was studied in a similar fashion. Thoracic aortae from 2 animals were cleaned and sectioned into eight 5 mm ring segments mounted between two pins and attached to a force displacement transducer (Gould, UC-3). Resting tension was 1.5 g, and the tissue was equilibrated for 60 min in normal PSS. The aortic segments were then incubated in the dark with and without calcium antagonists for 60 min, using 4 segments per pharmacological subset. The normal PSS was then exchanged for a calcium-free solution (containing 40 mM KCl, 0.5 mM EGTA, with and without calcium antagonists). Cumulative concentration-response curves were then generated as before. This technique produced a coefficient of variation in calcium curves of $< 10\%$.

In binding experiments, [^{3}H]-nitrendipine dissociation constant (K_D) and maximum binding (B_{max}) were determined as recommended by Munson & Rodbard (1983) and calculated from a computer-derived non-linear least squares program, MLAB (Knott, 1979), which was run on a DEC PDP-10 (Digital Equipment Corporation, Boston, Mass., U.S.A.) computer with access through the Prophet System (Rubin & Risley, 1977). Non-specific binding (B_{ns}) was determined from a linear fit and then substracted from the total binding curve that was modelled to fit the equation $B_{tot} = (B_{max} \times A/(K_D + A)) + mA + b$, where A = radioligand concentration, B_{tot} = total binding in c.p.m., B_{max} = maximum specific binding, K_D = radioligand dissociation constand and m and b are the slope and y intercept, respectively, of the non-specific binding curve. K_D and B_{max} were then determined by a fit of $B_{sp} = B_{max} \times A/K_D + A)$ where B_{sp} = specific binding, defined as B_{tot} – fitted B_{ns}, and B_{max} and – K_D are the asymptotes of the hyperbolic function. Curve weighting was as recommended by Rodbard & Feldman (1975).

K_i values were determined from the formula of Cheng & Prusoff (1973), $K_i = IC_{50}/(1 + A/K_D)$. The IC_{50} (concentration of antagonist that inhibits radioligand binding by 50%) and competition curve limits were determined by nonlinear least squares fit of the 4-parameter logistic equation of DeLean *et al.*, (1978).

Kinetic analysis was performed as described by Williams & Lefkowitz (1980). The association rate constant (k_1) was calculated from the formula $k_1 = (k_{obs} - k_2)/$ [[^{3}H]-nitrendipine]. k_{obs} was determined from the non-linear least squares fit of $B_{sp} = B_{eq}\ 1 - e^{-(k_{obs}t)}$ where B_{sp} = specifically bound [^{3}H]-nitrendipine at time t and $B_{eq} = B_{sp}$ at equilibrium. The dissociation rate constant k_2 was determined from the non-linear least squares fit of $B_{sp} = B_o e^{-k_2 t}$ where B_{sp} is the amount of specific binding remaining at time t from an initial amount B_o.

In tissue bath experiments a 'potency constant,' K_{CA}^{-1}, of calcium antagonists was calculated from assumptions based on previous work (Furchgott, 1967; Bristow & Green, 1977; Hurwitz *et al.*, 1982). The derivation of K_{CA}^{-1} is given in an appendix.

Nifedipine was dissolved in 95% ethanol as a 6 mM solution, with a subsequent 100 fold dilution in H_2O. Thereafter nifedipine dilutions were in 50 mM Tris buffer or PSS. All other calcium antagonists were prepared as 1 mM solutions in H_2O, and then diluted in buffer or PSS. [^{3}H]-nitrendipine, 88 Ci mmol^{-1}, was obtained from New England Nuclear, Boston, Massachusetts, U.S.A. and was used within 3 months of synthesis. Nifedipine was a gift from Pfizer Laboratories, New York, N.Y. Verapamil and D600 (methoxyverapamil) were obtained from AG Knoll, Ludwigshafen, West Germany. Diltiazem was supplied by Dr Ronald Brown of Marion Laboratories, Kansas City, Missouri. Nicardipine was supplied by Dr Arthur Strosberg of Syntex Corporation, Palo Alto, California. All other chemicals were purchased from Sigma Chemical Corporation, St. Louis, Missouri.

Statistical analysis was by Student's t test, with a $P < 0.05$ in the 2-tailed distribution taken as statistical significance.

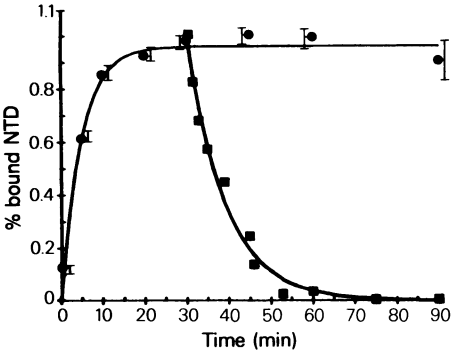

Figure 1 Association (●), and dissociation (■), kinetics of [^{3}H]-nitrendipine (NTD) in myocardial membranes. Dissociation kinetics were measured by adding 1 μM nicardipine (NCD) to membranes that had been incubated with 1.5 nM [^{3}H]-nitrendipine for 30 min. On the vertical axis 1.0 = 100% of specific [^{3}H]-nitrendipine binding. Association kinetics are means of 3 experiments, dissociation points are from 2 experiments with each point plotted individually. Vertical bars show s.e.mean.

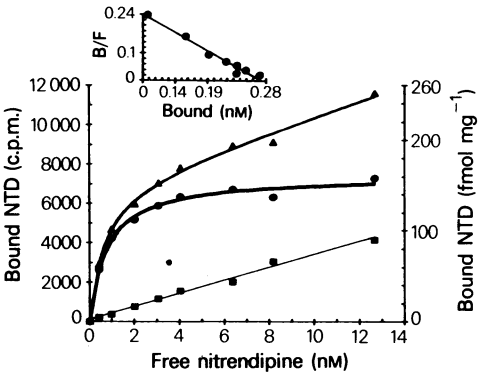

Figure 2 [^{3}H]-nitrendipine (NTD) binding in myocardial membranes, individual experiment. Data are expressed as counts per minute (c.p.m.) or fmol mg^{-1} protein relative to amount of free [^{3}H]-nitrendipine in assay tubes. (■) Non-specific binding, defined as [^{3}H]-nitrendipine bound in presence of 1 μM nicardipine; (▲) total [^{3}H]-nitrendipine bound; (●) specific [^{3}H]-nitrendipine binding (total − non-specific). Inset gives Scatchard plot for specific binding with B = bound and F = free. See Methods for further details.

Results

(1) *[^{3}H]-nitrendipine binding assay*

Specific [^{3}H]-nitrendipine binding in myocardial membranes was rapid and reversible, as shown in Figure 1. The k_1 for the data given in Figure 1 was 0.074 min^{-1} nM^{-1}, and the k_2 was 0.11 min^{-1}. The dissociation constant (k_2/k_1) calculated from these data was 1.49 nM, which is similar to the dissociation constant calculated from equilibrium binding experiments (Table 1). Specific [^{3}H]-nitrendipine binding was saturable (Figures 2 and 3), linear vs protein concentration in both myocardium and aorta (data not shown), and Scatchard analysis (Scatchard, 1949) yielded a single class of binding sites in both myocardium (Figure 2) and aorta.

Table 1 Summary of [^{3}H]-nitrendipine binding data

Tissue	*Maximum binding* (fmol mg^{-1})	*Maximum binding* (fmol g^{-1} wet weight)	K_D, (nM)
Heart	236.3 ± 29.9	8726.5 ± 536.2	1.05 ± 0.09
Aorta (n = 7)	59.3* ± 17.9	212.9* ± 31.2	1.56 ± 0.55

Values are means ± s.e.mean
*P < 0.001, compared to the values in the heart.

(2) *Receptor density and [^{3}H]-nitrendipine affinity data*

Figure 3 and Table 1 summarize [^{3}H]-nitrendipine binding data in aortic and myocardial membranes. In myocardium and aorta specific binding at a [^{3}H]-nitrendipine concentration of 0.5 nM averaged

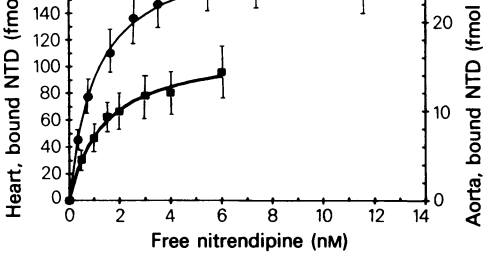

Figure 3 Mean specific binding isotherms for [^{3}H]-nitrendipine (NTD) in myocardial (●, n = 8) and aortic (■, n = 7) membranes. Vertical axes are normalized to fmol of [^{3}H]-nitrendipine per assay tube, i.e. per wet weight of tissue that yielded membrane protein for 1 tube (25 mg in myocardium and 125 mg in aorta). Verticals bars represent s.e.mean. Computer-fitted parameters are: Heart B_{max} = 190.3 fmol, K_D = 1.098 nM; aorta, B_{max} = 17.2 fmol, K_D = 1.382 nM. See Methods for further details.

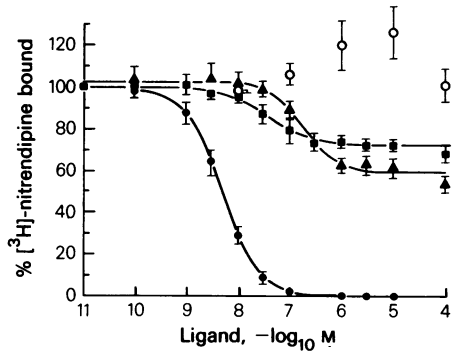

Figure 4 Effect of additions of nicardipine (●), D600 (■), verapamil (▲) and diltiazem (○) on specific [^{3}H]-nitrendipine binding in myocardial membranes, mean of 3 experiments. Specific [^{3}H]-nitrendipine binding is defined as that displaceable by 1 μM nicardipine, and was 89.6 ± 7.8% in these 3 experiments. On the vertical axis data are expressed as a % of maximum specific [^{3}H]-nitrendipine binding. Vertical bars represent s.e.mean. See Methods for further details.

89.5 ± 2.7% and 65.4 ± 4.1%, respectively. The [^{3}H]-nitrendipine B_{max} was much greater in eight myocardial preparations (Table 1). Mean K_D values were 1.05 ± .09 nM in myocardium and 1.56 ± .55 nM in aorta (*P* value *NS*).

(3) *Calcium antagonist–[^{3}H]-nitrendipine competition curves*

Figure 4 shows the interaction of 4 calcium antagonists with the [^{3}H]-nitrendipine binding site in myocardial membranes. Whereas nicardipine displaced around 90% of the [^{3}H]-nitrendipine bound, verapamil and D600 displaced < 50% of the bound [^{3}H]-nitrendipine. Diltiazem did not displace [^{3}H]-nitrendipine, and appeared to effect a small increase in [^{3}H]-nitrendipine binding at concentrations of 10^{-6} M and 10^{-5} M.

Figure 5 shows results of experiments in myocardial membranes in which nicardipine and nifedipine competition for [^{3}H]-nitrendipine binding was directly compared. As can be seen, nifedipine displaced [^{3}H]-nitrendipine in a manner similar to nicardipine, but was less potent. The mean *K*1 value for nicardipine was 2.19 ± .24 nM, compared to 7.90 ± 2.42 nM for nifedipine ($P < 0.05$).

Figure 6 shows experiments in which nicardipine competition curves were performed in myocardial and aortic membranes pooled from the same animals. As can be observed, nicardipine competition for the [^{3}H]-nitrendipine binding site is quite similar in the 2 membrane preparations. There was no statistically significant difference in the mean nicardipine K_i in heart and aorta (2.19 ± .24 nM vs 3.87 ± 1.08 nM, respectively). In addition, data for verapamil, D600 and diltiazem interaction with the [^{3}H]-nitrendipine binding site in aortic membranes were identical to data obtained in myocardial membranes.

(4) *Effect of Ca^{2+} on [^{3}H]-nitrendipine binding*

Additions of calcium gluconate had no effect on specific [^{3}H]-nitrendipine binding. Although non-specific [^{3}H]-nitrendipine binding progressively increased by 2.5 fold at 10^{-2} M calcium, Ca^{2+} had no effect on specific [^{3}H]-nitrendipine binding up to additions of 10^{-2} M.

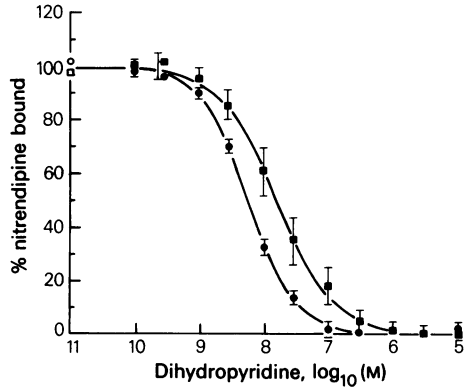

Figure 5 Competition of nicardipine (●) or nifedipine (■) for the [^{3}H]-nitrendipine myocardial binding site. % specific [^{3}H]-nitrendipine binding on vertical axis is expressed as a % of maximum. Results are mean ± s.e.mean (vertical bars) of 5 (nicardipine) and 3 (nifedipine) experiments. See Methods for further details.

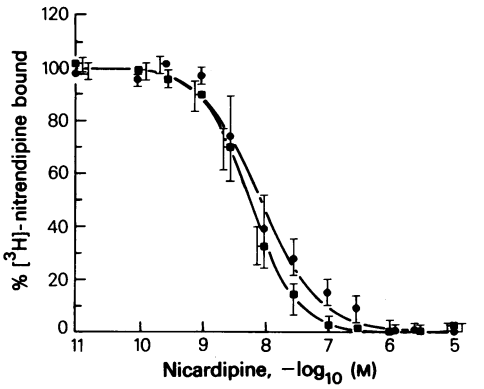

Figure 6 Competition of nicardipine (NCD) for the [^{3}H]-nitrendipine binding site in aorta (●) and heart (■). Results are mean ± s.e.mean of 5 (hearts) and 4 (aorta) grouped experiments.

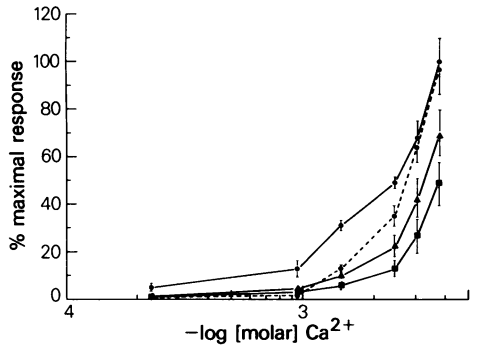

Figure 7 Effect of D600, verapamil or diltiazem on the concentration-response curve of Ca^{2+} in rabbit right ventricular strips.(●—●) Control, (■—■) plus D600 10^{-7} M, (▲—▲) plus verapamil 6×10^{-7} M and (●---●) plus diltiazem 10^{-6} M. Results are the mean ± s.e.mean (vertical bars) of 6–12 experiments with each antagonist, with all controls pooled together. Ca^{2+} was determined by ion-specific electrode. Addition of 10^{-3} M calcium chloride gives a Ca^{2+} of 9.4×10^{-4} M, and addition of 5×10^{-3} M calcium yields a Ca^{2+} of 3.75×10^{-3} M.

(5) *Tissue response*

In myocardium the inhibition of calcium concentration-response curves by 5 calcium antagonists is given in Figures 7 and 8 and Table 2. The order of potency of calcium antagonist inhibition of the Ca^{2+}-induced contractile response in myocardial tissue is D600 > verapamil > nifedipine > nicardipine > diltiazem. In myocardium calcium antagonists produced apparently parallel, rightward shifts of the calcium concentration-response curve. Because a bicarbonate buffer system was used, it was not generally possible to perform more than one shift of the dose-response curve to calcium, as Ca^{2+} precipitated from the bath at concentrations of > 3.75 mM. When we did attempt to perform 2 concentration-response curve shifts as for nicardipine in Figure 8, the shift at the lower concentration was not consistent, and large numbers of experiments were required to document a progressive rightward shift. In Figure 8, 2 concentrations of nicardipine do yield a progressive parallel rightward shift in the concentration-response curves, with similar K_{CA}^{-1} ('potency constant') values of 1.43×10^{-5} M and 8.57×10^{-6} M, respectively. The K_{CA}^{-1} value for 3×10^{-6} M nicardipine calculated from 21 experiments (and therefore having a slightly different control curve than that given in Figure 8) was $1.2 \pm 0.2 \times 10^{-5}$ M (Table 2). The potency of nicardipine and nifedipine in left atrial preparations was similar to data in ventricular strips (data not shown). Also, when calcium antagonists were progressively added in the presence of 1.25 mM Ca^{2+} and the IC_{50} calculated, similar degrees and orders of potency were noted.

In rabbit aortic ring segments (Figures 9 and 10, Table 2) calcium antagonists produced parallel or nearly parallel shifts of the calcium concentration-response curve. For diltiazem and nicardipine the effect on the calcium response simulated simple competitive antagonism, as shown in Figure 9. Additionally, single concentrations of D600, verapamil and nifedipine produced parallel or nearly parallel rightward shifts in the Ca^{2+} concentration-response curve (Figure 10). The order of potency was nicardipine > nifedipine > D600 > verapamil > diltiazem. In other words, for antagonism of Ca^{2+}-induced tissue response, myocardial tissue differs from aorta in that nicardipine is > 10,000 times more potent in vascular tissue. Nifedipine exhibited a 40 fold selectivity ratio in favour of vascular smooth muscle, despite the similarity of the nifedipine K_i in [^{3}H]-nitrendipine competition experiments in aorta and heart. Diltiazem also exhibited selectivity for vascular smooth muscle, as previously described (Ginsburg *et al.*, 1981).

Discussion

In this study, [^{3}H]-nitrendipine binding characteristics were qualitatively similar in membranes derived

Table 2 A comparison of the potencies (K_{CA}^{-1} values) of the various calcium antagonists for inhibiting contractile responses to Ca^{2+}

	Aorta		*Ventricle*	
D600	$4.0 \pm 0.3 \times 10^{-8}$	(10)	$2.2 \pm 0.2 \times 10^{-7}$	(6)
Diltiazem	$1.8 \pm 0.2 \times 10^{-7}$		$1.3 \pm 0.4 \times 10^{-5}$	(12)
	$*8.9 \times 10^{-8}$			
Verapamil	$1.6 \pm 0.3 \times 10^{-7}$	(8)	$4.9 \pm 1.8 \times 10^{-7}$	(8)
Nicardipine	$4.1 \pm 1.0 \times 10^{-10}$	(5)	$1.2 \pm 0.2 \times 10^{-5}$	(21)
	$*2.5 \times 10^{-10}$			
Nifedipine	$3.0 \pm 0.9 \times 10^{-9}$	(5)	$7.3 \pm 3.8 \times 10^{-7}$	(8)

The values shown are K_{CA}^{-1}, means ± s.e.mean. Numbers in parentheses refer to number of animals in each group.
Calcium antagonist concentrations are as given in Figures 8–10. In aorta for diltiazem and nicardipine * indicates K_{CA}^{-1} values are taken from pA_2 values, where $pA_2 = \log 1/K_B$ (Arunlakshana & Schild, 1959). For all other antagonists in aorta and myocardium $K_{CA}^{-1} = [A]/(DR - 1)$ was used (see Appendix). K_{CA}^{-1} values for nicardipine in myocardium are based on the 3×10^{-5} M concentration, in aorta nicardipine K_{CA}^{-1} are calculated for the 3×10^{-10} M concentration. In aorta K_{CA}^{-1} values for diltiazem are from the 10^{-6} M concentration.

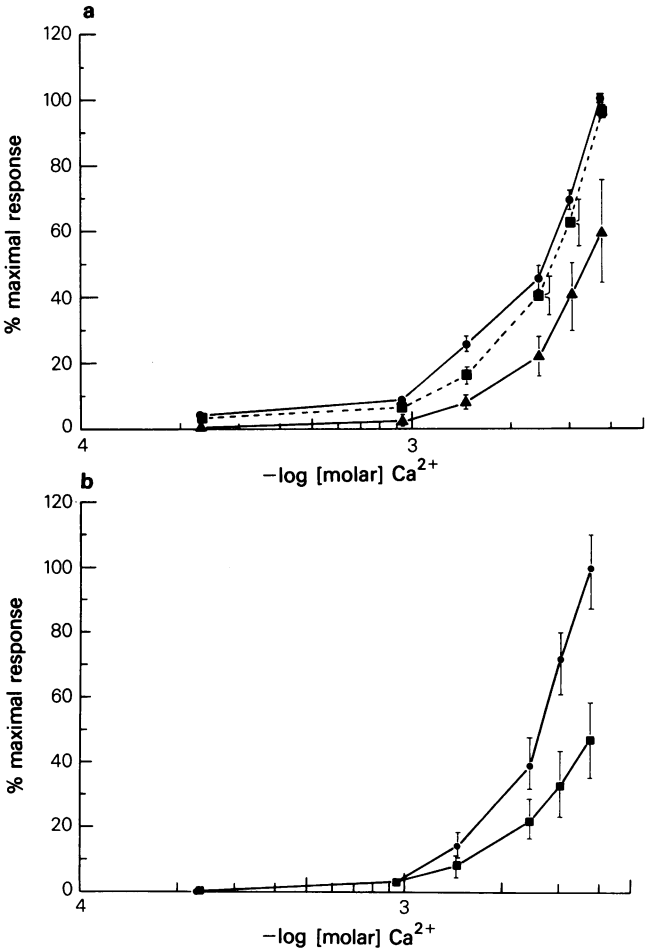

Figure 8 (a) Effect of nicardipine on Ca^{2+} concentration response relationships in rabbits right ventricular strips. (▲—▲) Nicardipine 3×10^{-6} M, $n = 21$; (■---■) nicardipine 10^{-6} M, $n = 44$; (●—●) control values are grouped together. See Figure 7 and Methods for further details. (b) Effect of nifedipine 10^{-7} M on the Ca^{2+} concentration-response curve in rabbit right ventricular strips, $n = 8$. (●—●) control, (■—■) plus nifedipine 10^{-7} M, $n = 8$. See Figure 7 and Methods for further details.

from rabbit ventricular myocardium and aorta. [^{3}H]-nitrendipine binding data were in general agreement with the data from other laboratories (Bellemann *et al.*, 1981; Bellemann *et al.*, 1982; Bolger *et al.*, 1982; Ehlert *et al.*, 1982a,b; Gould *et al.*, 1982; Murphy & Snyder, 1982; Williams & Tremble, 1982). The K_D of [^{3}H]-nitrendipine was 1.05 nM in myocardium and 1.56 nM in aorta, which is in the mid-range of values previously obtained in myocardium (Bellemann *et al.*, 1981; 1982; Murphy & Synder, 1982; Ehlert *et al.*, 1982a,b; Williams & Tremble, 1982). The number of [^{3}H]-nitrendipine binding sites was also similar to that previously found for myocardial membranes (Bellemann *et al.*, 1981; 1982; Murphy & Synder, 1982; Ehlert *et al.*, 1982a,b; Bolger *et al.*, 1982; Williams & Tremble, 1982). Finally, the interaction of verapamil and D600 with the [^{3}H]-nitrendipine binding site was similar to data described by others (Ehlert *et al.*, 1982a,b), as was the slight enhancement of [^{3}H]-nitrendipine binding by diltiazem (Depover *et al.*, 1982).

Unlike Murphy & Snyder (1982), we did not observe displacement by diltiazem of bound [^{3}H]-nitrendipine. Also, we did not observe any effect of calcium on [^{3}H]-nitrendipine binding, (Gould *et al.*, 1982; Ehlert *et al.*, 1982a,b), nor any differences between [^{3}H]-nitrendipine binding sites in vascular smooth muscle and myocardium, as was suggested by earlier investigations (Williams & Tremble, 1982; Depover *et al.*, 1982).

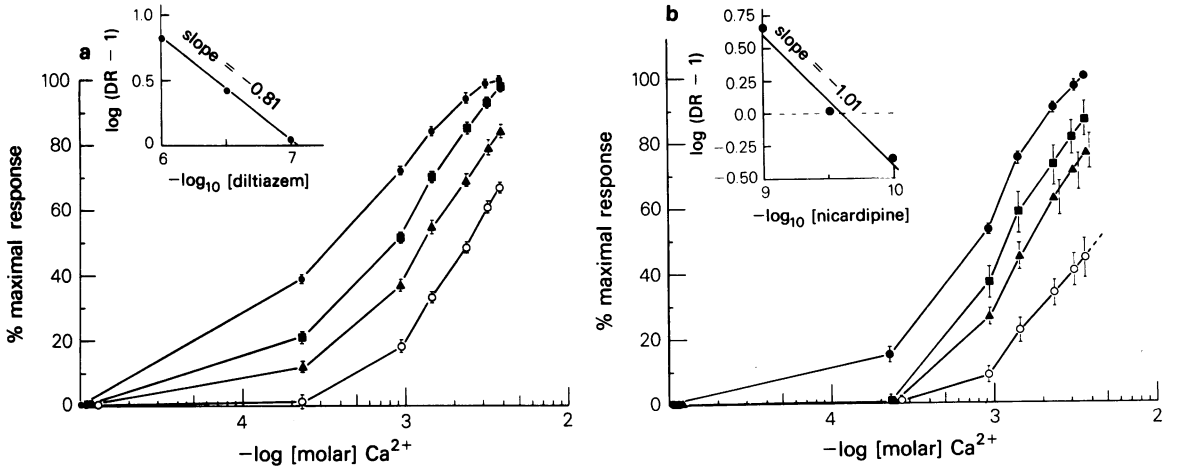

Figure 9 (a) Effect of increasing concentrations of diltiazem (control (●), diltiazem 10^{-7} M (■), 3×10^{-7} M ((▲), 10^{-6} M(○)) on the Ca^{2+} concentration-response curve in rabbit aorta ring segments. Inset gives Schild plot (Arunlakshana & Schild, 1959). Vertical bars show s.e.mean, $n = 6-10$, all controls were pooled. See Methods for further details.
(b) Effect of increasing concentrations of nicardipine (control (●), nicardipine 10^{-10} M (■), 3×10^{-10} M (▲), 10^{-9} M (○)) on the Ca^{2+} concentration-response curve in rabbit aortic ring segments. Inset gives Schild plot. Vertical bars show s.e.mean, $n = 5-6$, all controls were pooled.

Myocardial membranes had 4 times more [^{3}H]-nitrendipine binding sites than aortic membranes. This difference in binding site density is apparently not a product of the slightly different method of membrane preparation, as β-receptor density in our myocardial preparation is of the same order (53.1 ± 17.4 fmol mg^{-1} (mean ± s.d.) Lewis *et al.*, 1981) as α_1-adrenoceptor and H_1-histamine receptor densities in this aortic preparation (16.8 ± 5.9 and 71.1 ± 19.2 fmol mg^{-1}, respectively, using [^{125}I]-BE2254 and [^{3}H]-mepyramine, Bristow & Hoffman, unpublished observations). If the [^{3}H]-nitrendipine binding sites were linked to sarcolemmal Ca^{2+} flux and subsequent muscle contraction in a similar manner in heart and aorta, the increased density of these sites in myocardium would dictate a leftward shift of the Ca^{2+} concentration-response curve relative to that in the aorta. Ca^{2+} concentration-response curves measured in rabbit ventricular papillary muscles were actually to the right of similar curves for aortic ring segments, by approximately 4 fold. Thus the relative sensitivity of these tissues to Ca^{2+} is in the opposite direction to that predicted by quantification of putative calcium channels by [^{3}H]-nitrendipine binding. However, differences in the manner in which Ca^{2+} concentration-response curves were performed in cardiac and aortic tissue make such comparisons difficult. Moreover, there is no *a priori* reason why calcium channels should be linked to muscle contraction in the same way in aorta and myocardium.

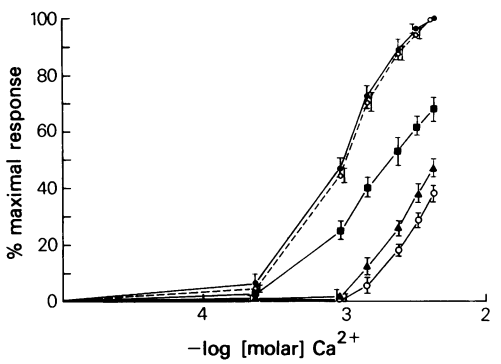

Figure 10 Effect of nifedipine 3×10^{-9} M (■, $n = 5$) D600 10^{-7} M (▲, $n = 8$) or verapamil 6×10^{-7} M (○, $n = 7$) on the Ca^{2+} concentration-response curve in rabbit aortic ring segments. (●—●) Controls for nifedipine experiments, (○---○) grouped controls for D600 and verapamil. Vertical bars show s.e.mean.

In myocardial tissue calcium antagonists shifted Ca^{2+} concentration-response curves to the right in a parallel or nearly parallel fashion, consistent with a competitive type of antagonism as previously described (Bristow & Green, 1977). Although in the current study competitive antagonism was not formally demonstrated in myocardium, the dihydropyridine compound nicardipine did produce a progressive parallel rightward shift of the calcium concentration-response curves in the two concentrations that could be evaluated, and these 2 concentra-

tions produced similar K_{CA}^{-1} values. However, it should be understood that in myocardium the K_{CA}^{-1} values derived are not true dissociation constants for calcium antagonists and their site of action. Rather, we used the 'potency constant' K_{CA}^{-1} as a measurement of the relative potencies of calcium channel blockers.

In aortic segments the greater sensitivity to Ca^{2+} allowed 3 progressive increases in concentration of the calcium antagonists diltiazem and nicardipine. Both of these compounds fulfilled criteria for competitive antagonism, with linear Schild plots with (−) slopes near unity. For one concentration of D600, verapamil and nifedipine, nearly parallel rightward shifts in the Ca^{2+} concentration-response curve were observed, but K_{CA}^{-1} values should be taken only as a measure of relative potency for these 3 antagonists.

The competitive antagonism of Ca^{2+} by calcium antagonists in rabbit aorta is in disagreement with previous studies with nifedipine (Schümann *et al.*, 1975; Godfraind, 1983), or D600 (Schümann *et al.*, 1975). However, in these previous studies dose-response curves were based on the amount of calcium added to the bathing medium rather than on measured ionized calcium (Ca^{2+}); with a bicarbonate buffer, addition of calcium causes progressive complexing of Ca^{2+} until precipitation of insoluble calcium salts occurs. Thus, in the higher concentration ranges curves will appear to be non-competitive when expressed as a function of added calcium, but may be competitive when expressed as a function of Ca^{2+}.

Our data reveal a discrepancy in the potency of nicardipine for competition at myocardial [^{3}H]-nitrendipine binding sites and antagonism of ventricular response. In radioligand competition experiments nicardipine was the most potent agent evaluated, with a K_i of 2.19 nM. However, μM amounts of nicardipine were required to antagonize ventricular responses to Ca^{2+}, a difference >3 orders of magnitude. In consequence, for [^{3}H]-nitrendipine binding in myocardium the order of potency was nicardipine $>$ nifedipine $>$ D600 $>$ verapamil $>$ diltiazem, and for ventricular response the order of potency was D600 $>$ verapamil $\geq$ nifedipine $>$ nicardipine $\geq$ diltiazem. The relative and absolute potencies of D600, verapamil, nifedipine and diltiazem are in agreement with those found previously in isolated myocardial tissues (Millard *et al.*, 1982).

Other differences between binding and tissue response are relatively minor. In aorta the order of potency for inhibition of [^{3}H]-nitrendipine binding and tissue response was similar and K_{CA}^{-1} values were in general agreement with K_i values. However, the K_{CA}^{-1} calculated for nicardipine was 5–9 times less than the K_i. Also, diltiazem did not displace [^{3}H]-nitrendipine from its binding site in either myocardium or aorta, whereas it did antagonize Ca^{2+}-mediated responses in both tissues. Finally, although tissue response data predicted competitive antagonism between Ca^{2+} and some calcium antagonists, the addition of Ca^{2+} to myocardial membrane preparations had no effect on [^{3}H]-nitrendipine binding.

Our data in rabbit myocardial and aortic membranes indicate that the [^{3}H]-nitrendipine binding site is identical in the 2 tissues. Differences in the characteristics of the [^{3}H]-nitrendipine binding site therefore do not explain tissue selectivity of the 1,4-dihydropyridine calcium antagonists. Although it is possible that differences in the methods employed to evaluate calcium antagonist potency in cardiac and aortic tissue could have led to the observed differences and artificially created tissue selectivity, for several reasons we believe this is unlikely. Firstly, the methods differed only in exposure of aorta to KCl depolarization. Other investigators (Siegl & McNeill, 1980; Mantelli *et al.*, 1981) have observed general agreement between calcium antagonist potency in depolarized and non-depolarized myocardium. Also, exposure of cardiac membranes to high concentrations of KCl has no effect on [^{3}H]-nitrendipine binding characteristics (unpublished observations), indicating that KCl depolarization does not irreversibly alter Ca^{2+} channel structure. Secondly, the order of calcium antagonist potency was different in myocardium and aorta; different antagonists would have to be affected to different degrees for methodology differences to account for the observed results. Finally, our results showing nicardipine and diltiazem selectivity for vascular response agrees with *in vivo* data (Satoh *et al.*, 1980; Walsh *et al.*, 1981).

The discrepancy between [^{3}H]-nitrendipine binding data and the physiological response is in agreement with a recent report by DePover *et al.* (1982), who found an order of magnitude difference between the [^{3}H]-nitrendipine K_D and the concentration at which the cold ligand inhibited contractile force in dog trabecular strips. These authors found comparable potencies for nitrendipine binding and inhibition of the physiological response in porcine coronary arteries, similar to our potency data in rabbit aorta. Although DePover *et al.* (1982) concluded that the nitrendipine binding site was different in porcine coronary artery and dog myocardium; this conclusion was based on a 14.3 fold difference in [^{3}H]-nitrendipine K_D in membranes prepared by two different techniques from tissue originating from two different species.

The above observations lead us to conclude that the selectivity of certain calcium antagonists is not due to differences in [^{3}H]-nitrendipine binding sites. Our data indicate that although [^{3}H]-nitrendipine binds to high affinity sites in heart and aorta, these

sites may be unrelated to a physiological calcium channel. At the very least, it appears that [^{3}H]-nitrendipine binding data do not directly correlate with the physiological effects of some calcium antagonists.

Appendix

Derivation of K_{CA}^{-1}, potency constant of a calcium antagonist, based on assumption that a calcium antagonist competes with calcium ion for a binding site (calcium channel) on the cell surface membrane*

$$[C] + n[Ca^{+2}_{E_0}] \underset{k_2}{\overset{k_1}{\rightleftarrows}} [Ca^{+2}_n C] \xrightarrow{k_3} [Ca^{+2}_I] \rightarrow R$$

$$+$$

$$m[A]$$

$k_4 \uparrow\downarrow k_5$ where

$[A_mC]$ C = [Calcium channels]

$Ca^{+2}_{E_0}$ = extracellular ionized calcium

n = number of molecules of Ca^{+2} combining with each C

Ca^{+2}_n = calcium bound to C

k_1, k_2, k_3 = rate constants

Ca^{+2}_I = intracellular ionized calcium

R = response (muscle contraction)

A = [unbound calcium antagonist]

m = number of molecules of A combining with each C

k_4, k_5 = rate constants

A_m = [Calcium antagonist] bound to C

*Since some but not all calcium antagonists have been shown to obey mass action laws for simple competition with C (Bristow & Green, 1977) the derived parameter K_{CA}^{-1} is understood to be useful as a measurement of the relative potency of calcium antagonists, and is not an apparent dissociation constant (K_B)

At equilibrium

without A

$k_1/(k_2 + k_3) = K_{Ca}{}^{+2} = Y/[Ca^{+2}_{E_0}]^n (1 - Y)$, where

(1) Y = proportion of C occupied by Ca^{2+}

$$= \frac{\{K_{Ca}{}^{+2}\}[Ca^{+2}_{Eo}]^n}{1 + \{K_{Ca}{}^{+2}\}[Ca^{+2}_{Eo}]^n}$$

In presence of A at same R (2) $Y = \{K_{Ca}{}^{+2}\}[Ca^{+2}_{E_A}]^n /$

$$(1 + K_{CA}[A]^m + \{K_{Ca}{}^{+2}\}[Ca^{+2}_{E_A}]^n)$$

where $Ca^{+2}_{E_A}$ is the extracellular Ca^{2+} when A is present

by definition (1) = (2);

simplifying and solving for K_{CA} gives

$$K_{CA} = \frac{[Ca^{+2}_{E_A}]^n - [Ca^{+2}_{Eo}]^n}{[Ca^{+2}_{Eo}]^n [A]^m}$$

$$= \frac{[Ca^{+2}_{E_A}]^n}{[Ca^{+2}_{Eo}]^n [A]^m} - \frac{[Ca^{+2}_{Eo}]^n}{[Ca^{+2}_{Eo}]^n [A]^m}$$

define $[Ca^{+2}_{E_A}] / [Ca^{+2}_{Eo}]$ as = to DR, the dose-ratio

$$K_{CA} = \frac{DR^n}{[A]^m} - \frac{1}{[A]^m}$$

$$= \frac{DR^n - 1}{[A]^m}$$

$$K_{CA}^{-1} = \frac{[A]^m}{DR^n - 1}$$

If one molecule of calcium antagonist interacts competitively with one calcium ion for occupation of one calcium channel ($m = 1$, $n = 1$) then the expression $\frac{[A]}{DR - 1} = K_{CA}^{-1}$. If simple competition is proven, this expression will be equivalent to an apparent dissociation constant. If competitive antagonism is not proven then this expression may be used as a measurement of the potency of a calcium antagonist, and as such can be termed a 'potency constant' equivalent to the concentration of a calcium antagonist that will shift a Ca^{2+} concentration-response curve 2 fold to the right.

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