[³H]-verapamil binding to rat cardiac sarcolemmal membrane fragments; an effect of ischaemia

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- 1 The [³H]-verapamil binding activity of rat cardiac sarcolemmal fragments was studied, using membranes harvested from non-perfused, aerobically-perfused and ischaemic hearts.
- 2 Glass-fibre filters were found to contain specific, high affinity (K_D 38 ± 3.1 nM) [³H]-verapamil binding sites—making them unsuitable for use in [³H]-verapamil binding studies.
- 3 Incubation of membranes from non-perfused hearts in a medium containing 150 mM NaCl, 1 mM CaCl₂ and 50 mM Tris revealed two populations of [³H]-verapamil binding sites. When centrifugation instead of filtration was used to separate bound and free [³H]-verapamil, high affinity sites with a K_D of 0.57 ± 0.19 μ M and a B_{max} of 38 ± 5.2 pmol mg⁻¹ protein, and low affinity sites with a K_D of 78 ± 27.5 μ M and a B_{max} of 2.9 ± 1.3 nmol mg⁻¹ protein were detected. However, only low affinity binding sites could be detected in membranes which had been incubated in a cation-free medium containing 50 mM Tris.
- 4 [³H]-verapamil binding to the low and high affinity sites was saturable, reversible, stereospecific and displaceable by D600 > diltiazem > Ca²⁺ but not by nifedipine, nitrendipine, nisoldipine or prazosin.
- 5 The two populations of binding sites survived aerobic perfusion and 60 min ischaemia at 37°C. Ischaemia reduced the B_{max} and K_D but selectivity was maintained.

Introduction

The Ca²⁺ antagonists (Ca²⁺ channel blockers) modulate slow channel function by interacting with specific, membrane-located sites (Bayer *et al.*, 1982; Lee & Tsien, 1983; Janis & Scriabine, 1983; Triggle & Janis, 1984; Schwartz & Triggle, 1984; Glossmann *et al.*, 1985). The binding sites for the dihydropyridine-based antagonists have been extensively investigated (Bellemann *et al.*, 1981; 1982; 1983; Janis *et al.*, 1982; Gould *et al.*, 1982; Williams & Tremble, 1982; Holck *et al.*, 1982; 1983; Murphy & Snyder, 1982; Ehlert *et al.*, 1982; DePover *et al.*, 1982; Bolger *et al.*, 1983; Fossett *et al.*, 1983; Sarmiento *et al.*, 1983; Janis & Triggle, 1984; Triggle & Janis, 1984; Striessnig *et al.*, 1985; Glossmann *et al.*, 1985), and two populations of binding sites have been identified: high affinity sites, with a dissociation constant (K_D) of 0.1–0.3 nM and a density (B_{max}) of 50–200 fmol mg⁻¹ protein, and low affinity sites, with a K_D of 60 nM and a B_{max} of 35 pmol mg⁻¹ protein. These binding sites are saturable and stereospecific, and the bound ligand—usually

[³H]-nitrendipine—is displaceable (Glossmann *et al.*, 1985).

As far as phenylalkylamine-based Ca²⁺ antagonists are concerned, their binding sites have been less thoroughly investigated. Hulten *et al.* (1982) detected a small number of high affinity [³H]-verapamil binding sites in frog hearts. By contrast, microsomal fractions harvested from skeletal muscle T-tubules (Reynolds *et al.*, 1983; Garcia *et al.*, 1984; Galizzi *et al.*, 1984a,b; Glossmann *et al.*, 1984) and cerebral cortex (Reynolds *et al.*, 1983) seem to contain a relatively large number of low affinity [³H]-verapamil binding sites.

The following studies were undertaken to characterize further the specific [³H]-verapamil binding sites in cardiac sarcolemmal fragments and to establish whether the binding activity of these sites is modified by sixty minutes of normothermic ischaemia.

Methods

Sarcolemmal membranes harvested from adult male Sprague-Dawley rats (250–300 g) were used. The rats

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were maintained on a standard diet, and tail cuff measurements showed them to be normotensive.

Perfusion sequence

The rats were lightly anaesthetized with a diethylether-O₂ mixture and treated with heparin. The hearts were then excised, immersed in ice-cold perfusion buffer and either immediately subfractioned or perfused in the Langendorff mode, as previously described (Nayler *et al.*, 1984). The perfusion buffer was a modified Krebs-Henseleit solution containing, in mM: NaCl 119.0, NaHCO₃ 25.0, KCl 4.6, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 1.3 and glucose 11.0. It was gassed with 95% O₂ + 5% CO₂ (pH 7.4), maintained at 37°C, and unless otherwise stated, delivered to provide a mean coronary flow of 10–12 ml min⁻¹. Spontaneously beating preparations was used to avoid any possibility of electrical stimulation releasing endogenous noradrenaline.

After 15 min aerobic perfusion the hearts were randomly divided into two groups. One group was perfused for another 60 min as described above. The other hearts were made globally ischaemic for 60 min, by totally occluding coronary flow whilst maintaining left ventricular wall temperature at 37°C, by means of temperature-controlled water-filled heating jackets.

Isolation of sarcolemmal membrane fragments

After the required period of aerobic perfusion or global ischaemia the hearts were immersed in ice-cold homogenizing medium containing 250 mM sucrose and 10 mM Tris HCl (pH 8.1). Freshly excised but unperfused hearts were treated in the same way. The ventricles were trimmed of atria, weighed and sarcolemmal membrane fragments isolated by differential centrifugation and sucrose gradient purification as described elsewhere (Daly *et al.*, 1984).

Characterization of membranes

The isolated membranes were enzymatically characterized in terms of their Na⁺/K⁺-ATPase (Daly *et al.*, 1984), 5' nucleotidase (Edwards & Maguire, 1970) and succinate dehydrogenase (Cooperstein *et al.*, 1950) activity and sialic acid content (Warren, 1959).

Radioligand binding assay

Equilibrium binding studies

[³H]-verapamil binding [³H]-verapamil binding was monitored for 30 min at 25°C in triplicate, using a reaction mixture containing 150 mM NaCl, 1.0 mM CaCl₂, 50 mM Tris (pH 7.4) and a protein concentration of 0.05–0.1 mg ml⁻¹ (Hulthen *et al.*, 1982).

Bound and free [³H]-verapamil were separated by filtration or centrifugation as indicated.

When separation was by filtration, 2–60 nM [³H]-verapamil was used in the absence or presence of 20 μM unlabelled verapamil for total and non-specific binding respectively. The reaction was terminated by adding 3.5 ml of ice-cold 50 mM Tris buffer (pH 7.4), followed immediately by vacuum filtration across Whatman GF/B filters. The filters were washed twice with 3.5 ml of 50 mM Tris buffer, (pH 7.4) and the filter radioactivity counted (40–45% efficiency) in Filter Count Scintillant, using a Packard Tricarb Spectrometer.

When centrifugation was used, 15 nM [³H]-verapamil, together with unlabelled verapamil over the concentration range 5 nM–1 mM was added for saturation, and 2 mM methoxyverapamil (D600) for non-specific binding. The whole reaction was carried out in siliconized Eppendorf tubes. After 30 min incubation at 25°C, the tubes were cooled on ice for 5 min before separating bound and free [³H]-verapamil by centrifugation at 11,800 g for 15 min at 4°C in a Hicracus Christ microfuge (model 918). The tubes were then placed on ice. Five min later the supernatant was carefully but quickly removed and the pellet immediately washed with 0.5 ml ice-cold 50 mM Tris (pH 7.4). The washing solution was immediately removed by vacuum suction and the radioactivity of the pellet established by scintillation counting as described above.

[³H]-nitrendipine binding To ensure that the sarcolemmal fragments used in these studies bound [³H]-nitrendipine as described by other investigators, [³H]-nitrendipine binding was characterized as outlined by Bellemann *et al.* (1981). Cardiac sarcolemmal membrane fragments were exposed to 0.05–12 nM [³H]-nitrendipine for 90 min in an incubation medium containing 150 mM NaCl, 1 mM CaCl₂, 50 mM Tris (pH 7.4). Unlabelled nitrendipine, 20 μM, was used to establish non-specific binding and the bound and free [³H]-nitrendipine were separated by vacuum filtration across Whatman GF/B filters. The filters were counted for radioactivity as described above.

Displacement studies The specificity of [³H]-verapamil binding was determined by standard displacement techniques. Thus 0.1 μM–10 mM CaCl₂, 1 nM–1 mM D600, 0.1 μM–1 mM diltiazem or 0.1 nM–100 μM nifedipine, nisoldipine, nitrendipine, or prazosin was added to membranes which had been pre-incubated with 15 nM [³H]-verapamil, together with either 45 nM or 60 μM unlabelled verapamil.

To establish whether the [³H]-verapamil binding was stereo-selective, (+)-verapamil (1 nM–1 mM) and (–)-verapamil (1 nM–1 mM) were used as displacers of bound [³H]-verapamil.

Protein determination

Protein content was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Analysis of data

Initial estimates of equilibrium binding parameters (K_D and B_{max}) were obtained from 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 weighed non-linear square computer curve fitting programme, 'LIGAND' (Munson & Rodbard, 1980). This was used to obtain final parameter estimates suitable for establishing whether the binding data best fitted a one or two site model – as assessed by the built in *F*-ratio test.

K_D is the equilibrium constant of the ligand for its receptor and is equal to the ratio of the forward rate constant versus the reverse rate constant. B_{max} is the maximum number of binding sites. K_i is the equilibrium dissociation constant for the interaction of the competing agent (I) with the receptor for the ligand (L). Thus

$$K_i = \frac{IC_{50}}{(1 + [L]/K_D)}$$

where the IC_{50} is the concentration of the competing ligand which displaces 50% of the bound ligand [L]. K_D refers to the dissociation constant of ligand (L).

The results are presented as mean $\pm$ s.e.mean of *n* determinations, the estimations for which were made in triplicate. Tests of significance were calculated by Student's *t* test taking $P < 0.05$ as the limit of significance.

Chemicals and reagents

[³H]-verapamil and [³H]-nitrendipine were obtained from New England Nuclear (Boston, Mass) as N-methyl-[³H]-verapamil hydrochloride and 5-methyl-[³H]-nitrendipine with a specific activity of 75.0 and 70.0 Ci mmol⁻¹ respectively.

Verapamil and methoxyverapamil (D600) were used either as the racemate hydrochlorides or as the (+)- and (–)-isomers, and were obtained as a gift from Knoll AG, Ludwigshafen, Germany. Nifedipine, nisoldipine and nitrendipine were obtained as gifts from Bayer AG, Germany, and diltiazem from Tanabe Laboratories, Japan. Prazosin-HCl was obtained as a gift from Pfizer Ltd, Sydney, Australia. Bovine serum albumin was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. All other reagents were from Sigma Chemical Company, St. Louis, USA.

Table 1 [³H]-verapamil binding to cardiac sarcolemmal membranes isolated from non-perfused, aerobically-perfused and ischaemic hearts, using filtration to separate bound and free [³H]-verapamil

Preparation	Incubation medium	K_D (nM)	B_{max} (pmol mg ⁻¹ protein)	Hill coefficient
<i>Non perfused hearts</i>				
	50 mM Tris	24.0 $\pm$ 7.8	28.5 $\pm$ 7.5	1.006 $\pm$ 0.012
	150 mM NaCl			
	1 mM CaCl ₂	39.0 $\pm$ 8.3	13.1 $\pm$ 6.6	1.012 $\pm$ 0.022
	50 mM Tris			
<i>In the absence of sarcolemmal membranes (filter blank)</i>				
	150 mM NaCl			
	1 mM CaCl ₂	38.0 $\pm$ 3.1	12.2 $\pm$ 1.9*	0.989 $\pm$ 0.05
	50 mM Tris			
<i>75 min aerobic perfusion</i>				
	150 mM NaCl			
	1 mM CaCl ₂	43.0 $\pm$ 7.6	15.3 $\pm$ 1.0	0.997 $\pm$ 0.07
	50 mM Tris			
<i>15 min aerobic perfusion + 60 min ischaemia</i>				
	150 mM NaCl			
	1 mM CaCl ₂	34.9 $\pm$ 6.4	14.8 $\pm$ 3.7	0.991 $\pm$ 0.05
	50 mM Tris			
		(NS)	(NS)	(NS)

*Denotes [³H]-verapamil binding in pmol per filter. Tests of significance relate to the significance of the difference in K_D , B_{max} and Hill coefficient caused by 60 min ischaemia, relative to the results obtained after aerobic perfusion. Each result is mean $\pm$ s.e.mean of 6 experiments. NS, not significant at $P < 0.05$.

Table 2 % of [^3H]-verapamil and [^3H]-nitrendipine bound to filters and centrifugation tubes

Ligand bound to	[^3H]-verapamil		[^3H]-nitrendipine	
	A	B	A	B
Filters	12.74	1.74	5.4	5.04
Non-siliconized tubes	1.80	0.41	3.16	1.89
Siliconized tubes	0.46	0.10	1.07	1.70

Total [^3H]-verapamil used: 32730 d.p.m., with specific activity $75.0 \text{ Ci mmol}^{-1}$. Total [^3H]-nitrendipine used: 28765 d.p.m., with specific activity $70.9 \text{ Ci mmol}^{-1}$. The reaction mixture contained 150 mM NaCl, 1 mM CaCl_2 and 50 mM Tris, pH 7.4. Incubation was for 30 min at 25°C in the absence (A) and presence (B) of $20 \mu\text{M}$ cold verapamil or nitrendipine. Each result is the mean of three separate experiments. Bound and free ligand were separated by filtration.

Results

[^3H]-nitrendipine binding

In the sarcolemmal membrane fragments used here two populations of [^3H]-nitrendipine binding sites were detected. The high affinity sites had a K_D of $0.23 \pm 0.04 \text{ nM}$ and a B_{max} of $258 \pm 87 \text{ fmol mg}^{-1}$ protein. The low affinity sites had a K_D of $31.7 \pm 5.5 \text{ nM}$ and a B_{max} of $11.9 \pm 1.2 \text{ pmol mg}^{-1}$ protein. The binding was saturable, reversible and displaceable, with bound [^3H]-nitrendipine being selectively displaced by nifedipine $> \text{D600}$.

[^3H]-verapamil binding to cardiac sarcolemmal membrane fragments

Equilibrium binding studies When cardiac membrane fragments prepared from freshly excised hearts were incubated in 50 mM Tris (pH 7.4), either in the absence or presence of 150 mM NaCl and 1 mM CaCl_2 , and the bound and free [^3H]-verapamil separated by filtration, a single population of high affinity [^3H]-verapamil binding sites was detected. Scatchard analysis of [^3H]-verapamil binding in membranes which had been incubated in 50 mM Tris without any added Na^+ or Ca^{2+} , revealed a single population of binding sites, with a K_D of $24 \pm 7.8 \text{ nM}$ and B_{max} of $28.5 \pm 7.5 \text{ pmol mg}^{-1}$ protein (Table 1). In the presence of Na^+ and Ca^{2+} , there was a significant ($P < 0.05$) reduction in the B_{max} ($13.1 \pm 6.6 \text{ pmol mg}^{-1}$ protein) of the binding sites but the affinity (K_D $39 \pm 8.3 \text{ nM}$) remained unchanged (Table 1).

When similar incubations were made but without any membrane fragments, and the incubation medium was 'filtered' exactly as described above, Scatchard analysis indicated that the [^3H]-verapamil had bound to the glass filters with a K_D of $38 \pm 3.1 \text{ nM}$ and a B_{max} of $12.2 \pm 1.9 \text{ pmol per filter}$ (Table 1). These values are close to those (Table 1) obtained for [^3H]-verapamil-

Figure 1 Scatchard plot of [^3H]-verapamil binding to rat isolated cardiac sarcolemmal membrane fragments incubated in (a) 50 mM Tris and (b) 150 mM NaCl, 1 mM CaCl_2 , 50 mM Tris, pH 7.4 at 25°C for 30 min. Membrane bound and free [^3H]-verapamil were separated by centrifugation. The binding parameters are given in Table 3. Ordinate scales: bound/free [^3H]-verapamil; abscissa scales: bound [^3H]-verapamil (nmol mg^{-1} protein). Similar estimates were obtained from six separate experiments, using triplicate estimates for each point.

membrane binding. Table 2 shows that in contrast to [³H]-nitrendipine, the [³H]-verapamil which binds to GF/B filters is displaceable by unlabelled verapamil. Under these conditions, therefore, data obtained from binding studies in which bound and free [³H]-verapamil have been separated by filtration across GF/B filters does not necessarily provide reliable data relating to the binding of [³H]-verapamil to sarcolemmal membrane fragments. Because of this, and since [³H]-verapamil binds with relatively low affinity (Reynolds *et al.*, 1983; Glossmann *et al.*, 1984; Garcia *et al.*, 1984; Galizzi *et al.*, 1984a or b) centrifugation (Bennett, 1978) rather than filtration seemed to provide a more appropriate method for separating bound and free [³H]-verapamil. Table 2 shows that the background binding of [³H]-verapamil to Eppendorf tubes is relatively small when compared to that bound by the filters and that siliconization of the tubes further reduces this binding.

When sarcolemmal membranes were incubated in 50 mM Tris (pH 7.4) and the bound and free [³H]-verapamil separated by centrifugation, only low affinity [³H]-verapamil binding sites, with a K_D of $68 \pm 5.5 \mu\text{M}$ and a B_{max} of $29.1 \pm 5.5 \text{ nmol mg}^{-1} \text{ nmol}$

mg^{-1} protein, were detected (Figure 1a and Table 3). However, adding 150 mM NaCl and 1 mM CaCl_2 to the reaction mixture, and separating bound and free ligand by centrifugation, provided evidence of two populations of binding sites (Figure 1b and Table 3)—a population of relatively high affinity sites, with a K_D of $0.57 \pm 0.19 \mu\text{M}$ and a B_{max} of $38 \pm 5.2 \text{ pmol mg}^{-1}$ protein, and a population of lower affinity sites, with a K_D of $78 \pm 27.5 \mu\text{M}$ and a B_{max} of $2.9 \pm 1.37 \text{ nmol mg}^{-1}$ protein (Table 3).

Displacement studies Studies in which 15 nM [³H]-verapamil and 45 nM unlabelled verapamil were added to membranes incubated in 150 mM NaCl, 1 mM CaCl_2 and 50 mM Tris and the bound and free [³H]-verapamil separated by filtration, showed that D600 displaced bound verapamil with a K_i of $2.4 \pm 0.6 \mu\text{M}$, and that neither nitrendipine, nisoldipine, nifedipine, Ca^{2+} , prazosin nor diltiazem were effective.

In contrast, under the same conditions of incubation but using centrifugation to separate bound and free [³H]-verapamil, Ca^{2+} , diltiazem and D600 displaced bound [³H]-verapamil with K_i values of 465 ± 15 , 14.6 ± 3.82 and $4.1 \pm 1.61 \mu\text{M}$, respectively

Table 3 [³H]-verapamil binding to cardiac sarcolemmal membranes isolated from non-perfused, aerobically perfused and ischaemic hearts, using centrifugation to separate bound and free [³H]-verapamil

Reaction mixture	High affinity			K_D (μM)	Low affinity	
	K_D (μM)	B_{max} (pmol mg^{-1} protein)	Hill coefficient		B_{max} (nmol mg^{-1} protein)	Hill coefficient
<i>Membranes prepared from non-perfused hearts</i>						
507 mM Tris	—	—	—	68.0 ± 5.5	29.1 ± 5.5	0.996 ± 0.004
150 mM NaCl						
1 mM CaCl_2	0.57 ± 0.19	38.00 ± 5.20	0.997 ± 0.008	78.00 ± 27.50	2.90 ± 1.30	0.993 ± 0.007
50 mM Tris						
<i>Membranes prepared after 75 min aerobic perfusion</i>						
150 mM NaCl						
1 mM CaCl_2	0.37 ± 0.10	22.36 ± 7.22	0.993 ± 0.002	74.86 ± 17.90	2.84 ± 0.77	0.995 ± 0.004
50 mM Tris						
<i>Membranes prepared after 15 min aerobic perfusion and 60 min ischaemia</i>						
150 mM NaCl						
1 mM CaCl_2	0.17 ± 0.04	3.88 ± 0.01	0.997 ± 0.01	34.45 ± 10.23	0.81 ± 0.39	0.970 ± 0.023
50 mM Tris						
	($P < 0.05$)	($P < 0.02$)	(NS)	($P < 0.03$)	($P < 0.02$)	(NS)

Tests of significance relate to the significance of the changes in K_D , B_{max} and Hill coefficient caused by 60 min ischaemia, relative to the values obtained after aerobic perfusion. Each result is mean $\pm$ s.e.mean of 6 experiments. NS, not significant at $P < 0.05$.

Figure 2 Displacement by Ca^{2+} (▲), diltiazem (●), D600 (■), nifedipine (◇), nitrendipine (□), nisoldipine (Δ) and prazosin (○) of $[^3\text{H}]$ -verapamil bound to sarcolemmal membranes isolated from non-perfused hearts. Membranes were incubated in 150 mM NaCl, 1 mM CaCl_2 and 50 mM Tris (pH 7.4) at 25°C for 30 min with 15 nM $[^3\text{H}]$ -verapamil and either (a) 45 nM cold verapamil (conditions suitable for high affinity binding), or (b) 60 μM cold verapamil conditions suitable for low affinity binding. Bound and free $[^3\text{H}]$ -verapamil were separated by centrifugation. The displacement parameters are given in the text. Ordinate scales: % bound; abscissa scales: drug concentration (log M). Similar estimates were obtained from 3–4 separate experiments, using triplicate estimates for each point.

(Figure 2). Again, neither the dihydropyridines nor prazosin displaced the bound $[^3\text{H}]$ -verapamil (Figure 2). Using 15 nM $[^3\text{H}]$ -verapamil and 60 μM cold verapamil, (conditions that are suitable for low affinity binding) bound $[^3\text{H}]$ -verapamil was displaceable by Ca^{2+} (K_i , $3940 \pm 420 \mu\text{M}$), diltiazem (K_i , $104 \pm 6.3 \mu\text{M}$), and D600 (K_i , $63.9 \pm 1.5 \mu\text{M}$) but not by nifedipine, nisoldipine, nitrendipine nor prazosin (see Figure 2).

Experiments in which the separate (+)- and (–)-isomers of verapamil were used to displace bound $[^3\text{H}]$ -verapamil revealed stereospecificity, with a K_i of $10.2 \pm 3.9 \mu\text{M}$ for the (+)-isomer and $4.1 \pm 1.2 \mu\text{M}$ for the (–)-isomer, for the high $36.4 \pm 15.2 \mu\text{M}$ and $6.4 \pm 1.2 \mu\text{M}$ for the low affinity sites.

$[^3\text{H}]$ -verapamil binding to sarcolemmal membranes harvested from aerobically perfused hearts

Experiments in which $[^3\text{H}]$ -verapamil binding was monitored by using centrifugation to separate bound and free $[^3\text{H}]$ -verapamil, revealed two populations of

$[^3\text{H}]$ -verapamil binding sites in sarcolemmal fragments harvested from aerobically perfused hearts (Figure 3). These were high affinity sites – with a K_D of $0.37 \pm 0.10 \mu\text{M}$ and a B_{max} of $22.36 \pm 7.22 \text{ pmol mg}^{-1}$ protein, and low affinity sites – with a K_D of $74.86 \mu\text{M}$ and a B_{max} of $2.84 \pm 0.77 \text{ nmol mg}^{-1}$ protein (Table 3). Bound $[^3\text{H}]$ -verapamil was displaceable by diltiazem (with a K_i of $2.58 \pm 0.91 \mu\text{M}$ for the high and $69.5 \pm 8.5 \mu\text{M}$ for the low affinity sites) and by D600 ($3.55 \pm 1.95 \mu\text{M}$ and $60.3 \pm 7.5 \mu\text{M}$ for the high and low affinity sites, respectively). Nitrendipine, however, did not displace bound $[^3\text{H}]$ -verapamil.

Effect of ischaemia on $[^3\text{H}]$ -verapamil binding

Separation by centrifugation Sarcolemmal membranes prepared from hearts which had been ischaemic for 60 min at 37°C retained two populations of $[^3\text{H}]$ -verapamil binding sites (Figure 3). The K_D of the high affinity sites was significantly reduced (by 54% ($P < 0.05$)) relative to the values obtained after aerobic perfusion, and there was an 82.7% ($P < 0.02$)

Figure 3 Scatchard plot for $[^3\text{H}]$ -verapamil binding to cardiac sarcolemmal membranes fragments isolated from aerobically perfused ($\blacktriangle$, 75 min aerobic perfusion) and globally ischaemic ($\triangle$, 15 min aerobic perfusion + 60 min ischaemia) hearts. Membranes were incubated in 150 mM NaCl, 1 mM CaCl_2 and 50 mM Tris (pH 7.4) at 25°C for 30 min. Membrane bound and free $[^3\text{H}]$ -verapamil were separated by centrifugation. The curves were fitted by non-linear regression analysis. The binding parameters are given in Table 3. Ordinate scale: bound/free $[^3\text{H}]$ -verapamil ($\times 10^{-2}$); abscissa scale: bound $[^3\text{H}]$ -verapamil (nmol mg^{-1} protein). Similar estimates were obtained from six separate experiments, using triplicate estimates for each point.

reduction in the B_{max} (Table 3), relative to the values obtained after aerobic perfusion. The K_D and the B_{max} of the low affinity sites were also altered, the K_D being reduced by 54% ($P < 0.03$) and the B_{max} by 71.5% ($P < 0.02$) (Table 3). However, the binding sites retained their selectivity; bound $[^3\text{H}]$ -verapamil being displaced by diltiazem (high affinity sites: K_i $0.37 \pm 0.03 \mu\text{M}$; low affinity sites: K_i $32 \pm 0.06 \mu\text{M}$) and D600 (K_i $1.36 \pm 0.79 \mu\text{M}$ and $32.2 \pm 3.8 \mu\text{M}$ for high and low affinity sites respectively) but not by nitrendipine. In summary, therefore, 60 min ischaemia significantly alters the $[^3\text{H}]$ -verapamil binding activity of the sarcolemma but the specificity of binding sites remains unchanged.

To establish whether this ischaemia-induced change in the activity of the verapamil binding sites is due to an effect of ischaemia (or some consequence of it) and not simply an artifact due to an altered protein content, or the isolation of a different subcellular fraction, the sarcolemmal fragments obtained from the ischaemic and aerobically perfused hearts were characterized. The results (Table 4) show that the yield

Table 4 Sarcolemmal profile

Perfusion	Aerobic	Ischaemic
Yield (mg protein g^{-1} heart wt)	0.60 ± 0.02	0.58 ± 0.04 (NS)
Sialic acid content (nmol mg^{-1} protein)	126.3 ± 4.7	147.7 ± 1.7 ($P < 0.01$)
$\text{Na}^+ \text{K}^+ \text{-ATPase}$ (ouabain sensitive) ($\mu\text{mol P}_i \text{mg}^{-1}$ protein h^{-1})	19.0 ± 1.1	11.3 ± 0.5 ($P < 0.01$)
5' nucleotidase ($\mu\text{mol P}_i \text{mg}^{-1}$)	14.0 ± 0.6	11.5 ± 0.7 ($P < 0.05$)
Succinate dehydrogenase ($\mu\text{mol cytochrome C}$ oxidized mg^{-1} protein h^{-1})	2.94 ± 0.2	3.04 ± 0.3 (NS)

Each result is mean $\pm$ s.e.mean of six separate experiments. Tests of significance relate to the significance of the change caused by ischaemia ($P < 0.05$) with respect to controls (aerobically perfused series). NS, not significant.

of sarcolemma and the presence of subcellular contamination, as gauged by succinate dehydrogenase activity, was not significantly altered. At the same time, however, there was a significant reduction in the activity of ouabain sensitive ATPase and 5' nucleotidase enzymes, and a significant increase in sialic acid content (Table 4).

Separation by filtration In the ischaemia $[^3\text{H}]$ -verapamil binding experiments described so far, bound and free $[^3\text{H}]$ -verapamil were separated by centrifugation. In another series of experiments in which only high affinity $[^3\text{H}]$ -verapamil binding sites were being monitored and where the bound and free $[^3\text{H}]$ -verapamil could therefore be separated by filtration, 60 min normothermic ischaemia did not appear to have any significant effect on $[^3\text{H}]$ -verapamil binding sites (Table 1). Table 1 shows that under these conditions only a single population of high affinity $[^3\text{H}]$ -verapamil binding sites, with a K_D of $43.0 \pm 7.6 \text{ nM}$ and B_{max} of $15.3 \pm 1.0 \text{ pmol mg}^{-1}$ protein, could be detected in membrane fragments isolated from aerobically perfused hearts. After 60 min ischaemia at 37°C , comparable sarcolemmal membrane fragments still contained a single population of high affinity $[^3\text{H}]$ -verapamil binding sites, with a K_D of $34 \pm 6.5 \text{ nM}$ and a B_{max} of $14.8 \pm 3.7 \text{ pmol mg}^{-1}$ protein (Table 1). Hence, when filtration was used to separate bound and free $[^3\text{H}]$ -verapamil 60 min ischaemia did not seem to have any significant effect on the high affinity $[^3\text{H}]$ -verapamil binding sites. This

contrasts with the data obtained when centrifugation was used to separate the bound and free ligand (Table 3).

Discussion

These results show that enzymatically characterized sarcolemmal membranes which contain high and low affinity [^3H]-nitrendipine binding sites, also contain two populations of [^3H]-verapamil binding sites. These are high affinity sites – with a K_D of $0.57\ \mu\text{M}$ and a B_{max} of $38\ \text{pmol mg}^{-1}$ protein, and low affinity sites – with a K_D of $78\ \mu\text{M}$ and a B_{max} of $2.9\ \text{nmol mg}^{-1}$ protein, respectively. The high affinity sites could only be detected in membranes which had been incubated in the presence of cations. Both the low and high affinity [^3H]-verapamil binding sites were saturable and stereospecific. Moreover the bound [^3H]-verapamil was displaceable by $\text{D600} > \text{diltiazem} > \text{Ca}^{2+}$ but not by nifedipine, nitrendipine or nisoldipine nor by the α_1 -adrenoceptor blocker, prazosin.

In the present study when glass fibre filters were used to separate bound and free [^3H]-verapamil, only a single population of relatively high affinity [^3H]-verapamil binding sites could be detected. Hulthen *et al.* (1982) Reynolds *et al.* (1983), Galizzi *et al.* (1984a,b), Glossmann *et al.* (1984) and Garcia *et al.* (1984) were also only able to detect a single population of high affinity sites (K_D 4–50 nM; B_{max} 50 fmol–13 pmol mg^{-1} protein) when they separated bound and free [^3H]-verapamil across glass fibre filters. However, the present results indicate that it is difficult to interpret the binding data obtained under these conditions because [^3H]-verapamil binds to glass fibre filters with an affinity and density comparable with that exhibited by membranes. Another factor which must be taken into consideration when selecting the most appropriate method for separating bound and free [^3H]-verapamil revolves around the relatively low affinity of [^3H]-verapamil binding. For radioligand binding studies in which the affinity of the ligand (K_D) exceed $10^{-8}\ \text{M}$, the recommended method for separating bound and free ^3H -ligand is by centrifugation (Bennett, 1978). It was for this reason that we used this method to characterize low affinity [^3H]-verapamil binding sites. If instead of centrifugation, separation is by filtration then because the ligand dissociates from its binding sites at a relatively rapid rate when the filters are washed, it is difficult to obtain a true estimate of K_D and B_{max} . These methodological problems could explain why some investigators have only been able to detect a single population of [^3H]-verapamil binding sites in isolated membranes.

The present results show that even when centrifugation was used to separate bound and free [^3H]-verapamil only a single population of low affinity [^3H]-

verapamil binding sites was detected when the membranes were incubated in 50 mM Tris. However, the inclusion of 150 mM NaCl and 1 mM CaCl_2 uncovered the second population of higher affinity binding sites. Galizzi *et al.* (1984a) have also described this second population of [^3H]-verapamil binding sites. Additional experiments are needed to explore this cation requirement of the high affinity sites. Presumably the low affinity [^3H]-verapamil binding cannot be attributed to a random accumulation of the ligand into the lipid bilayer, because [^3H]-verapamil binding at the low, as at the high, affinity sites was saturable, stereospecific and displaceable. It is reasonable to assume therefore that the binding is specific and that we are dealing with two populations of binding sites, with the B_{max} of the low affinity sites greatly exceeding that of the high affinity sites. However, each group (high and low affinity) contains a single population of sites, with a Hill coefficient approaching unity (Table 3).

The bound [^3H]-verapamil was not displaceable by the dihydropyridines or by prazosin. This inability of the dihydropyridines to displace [^3H]-verapamil cannot be attributed to an inability of the membranes to bind these compounds because both low and high affinity [^3H]-nitrendipine binding sites were present. It must be assumed, therefore, that the [^3H]-verapamil binding sites are distinct and separate from those occupied by the dihydropyridines, a finding which supports the conclusion reached by others (DePover *et al.*, 1982; Ferry & Glossmann, 1982; Millard *et al.*, 1983). The failure of prazosin to displace bound [^3H]-verapamil is surprising, because verapamil interacts with α_1 -adrenoceptors (Glossmann *et al.*, 1980; Nayler *et al.*, 1982; Karliner *et al.*, 1982). One possible explanation for this could be that only a small proportion of the [^3H]-verapamil binding sites is associated with α_1 -adrenoceptors. If this is indeed true, then any displacement of [^3H]-verapamil bound to these sites may be masked by a large population of [^3H]-verapamil binding sites not associated with these receptors.

Although our results show that cardiac sarcolemmal fragments contain both low and high affinity [^3H]-verapamil binding sites, and that these sites are distinct from those occupied by the dihydropyridines, the question which remains to be resolved relates to the physiological significance of the two populations of binding sites. The K_D of the high affinity sites falls within the concentration range which attenuates slow channel function (Kohlhardt *et al.*, 1975). By contrast, the K_D of the low affinity sites falls within the range at which verapamil acts as a local anaesthetic (Fairhurst *et al.*, 1980), blocks the fast inward Na^+ and slow outward K^+ currents (Kass & Tsien, 1975) and interacts with α -adrenoceptors (Glossmann *et al.*, 1980; Nayler *et al.*, 1982; Karliner *et al.*, 1982),

muscarinic and opiate receptors (Cavey *et al.*, 1977).

Our results show that sarcolemmal membranes which had been prepared from ischaemic hearts retained two populations of [³H]-verapamil binding sites. Ischaemia did, however, cause a significant decrease in the density (B_{max}) and dissociation constant (K_D) of both the high and low affinity binding sites, but their selectivity was unchanged. This effect of ischaemia is not peculiar to the phenylalkylamine binding sites. The dihydropyridine binding sites behave in the same way (Nayler *et al.*, 1985). Reports have appeared in the literature describing a lack of or opposite effects of ischaemia on other receptor systems, e.g. unaltered muscarinic and increased β -adrenoceptor numbers after 60 min ischaemia (Mukherjee *et al.*, 1979), and increased α_1 -adrenoceptor numbers after 30 min ischaemia (Corr *et al.*, 1981). Our inability to detect any effect of ischaemia on the profile of the high affinity binding sites, when the bound and free ligand was separated by filtration, probably reflects the difficulties that we encountered in obtaining true saturation kinetics under these conditions, because of the presence of high affinity [³H]-verapamil binding sites in the filters.

The present results show that although 75 min of aerobic perfusion failed to cause a significant change in either the K_D or B_{max} of the [³H]-verapamil binding sites being studied here, there was a trend towards a decrease in both the K_D and B_{max} . Despite this trend the overall specificity of the binding sites was retained, with nitrendipine failing to displace bound [³H]-verapamil in both instances. It is worth noting, however, that the altered ability of diltiazem (as indicated by the changed K_i), but not of D600, to displace bound [³H]-verapamil from membranes obtained from aerobically perfused relative to non-

perfused hearts is indicative of the fact that aerobic perfusion does have an effect on the diltiazem recognition sites. Whether prolonged periods of aerobic perfusion, in excess of the 75 min used here, alters the ability of other phenylalkylamines to displace bound [³H]-verapamil, requires further investigation.

An apparent hypersensitivity to verapamil has been reported for the ischaemic myocardium (Smith *et al.*, 1976; Smith & Briscoe, 1982). This hypersensitivity cannot be explained in terms of an ischaemia-induced increase in the number of verapamil binding sites, because the present results show that ischaemia reduces their number. However, we did find an increase in affinity associated with this decline in the number of binding sites. Possibly the affinity of the ligand for its binding site is functionally more important than the actual number of binding sites. Alternatively the hypersensitivity of the ischaemic myocardium to verapamil may involve mechanisms other than an increase in receptor number or affinity.

In conclusion, this study shows that the binding of [³H]-verapamil to cardiac sarcolemmal fragments is complex. Two populations of binding sites are involved, and the binding is cation sensitive, saturable, reversible, stereoselective and displaceable by D600 > diltiazem > Ca²⁺ but not by the dihydropyridines nor by prazosin. These binding sites survive a prolonged period of normothermic ischaemia, but with a reduced B_{max} and K_D . Their response to ischaemia therefore, resembles that of the dihydropyridine binding sites (Nayler *et al.*, 1985).

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