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THE EFFECT OF HYPOXIA AND OF ENERGY DEPLETION ON 1,4-DIHYDROPYRIDINE BINDING SITES IN RAT CARDIAC MEMBRANE FRAGMENTS

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Abstract—The effect of hypoxia, reoxygenation and chemically-induced high energy phosphate depletion (caused by inhibition of oxidative phosphorylation and glycolysis) on the affinity (K_d), density (B_{max}) and selectivity of high affinity 1,4-dihydropyridine (DHP) calcium antagonist binding sites was studied in rat isolated cardiac membranes, using (+)[^{3}H]PN200-110. Neither 30 nor 60 min normothermic (37°) hypoxia affected either the B_{max} or K_d of these sites, relative to aerobic controls. Fifteen min reoxygenation after 60, but not 30, min hypoxia reduced the density of the DHP binding sites, without altering their affinity or selectivity.

Aerobic perfusion with 0.1 mM DNP (an uncoupler of oxidative phosphorylation) for 30 min at 37° caused an increase in B_{max} ($P < 0.05$) both in the presence (48%) and absence (27%) of glucose, without any change in K_d. This increase in B_{max} was attenuated during a further 30 min perfusion with DNP. Thirty min perfusion with 1.0 mM IAA and 0.1 mM DNP resulted in a significant increase (27%) in the B_{max} of the DHP binding sites. A further 30 min perfusion with IAA and DNP caused the B_{max} to return to control levels. The K_d was not altered under these conditions. Irrespective of the perfusion conditions, the selectivity of the binding sites was unchanged, with (+)PN200-100 > (−)Bay K8644 > (−)PN200-110 = (+)Bay K8644 in displacing bound (+)[^{3}H]PN200-110. Under all conditions, (−)D600 always interacted allosterically with the DHP binding sites, and the binding was stimulated by d-*cis* diltiazem.

These results show that neither hypoxia nor chemically-induced ATP depletion mimic the effect of ischaemia on cardiac DHP binding sites.

Global ischaemia reduces the density (B_{max}), without altering either the affinity (K_d) or selectivity, of the dihydropyridine (DHP) binding sites in rat cardiac membrane fragments [1, 2]. Gu *et al.* [2] found that under normothermic conditions this ischaemia-induced reduction in B_{max} reaches asymptote within 30 min, and that reperfusion after 30, but not 60, min of ischaemia causes the B_{max} to return to its pre-ischaemic level. Maintaining the hearts at 22° rather than 37° during the ischaemic episode prevents the ischaemia-induced reduction in the density of these binding sites, even during a prolonged period of global ischaemia [2].

Although these preliminary studies establish that ischaemia reduces the density of the DHP binding sites, they provide little information as to the mechanisms which may be involved. Theoretically, the ischaemia-induced reduction in O_2 availability, the attendant depletion of the high energy phosphate stores (adenosine triphosphate (ATP) and creatine phosphate (CP)) [3, 4], and the resultant intracellular acidosis [5] might all be involved. The present study was undertaken to establish whether O_2 lack—as in hypoxia—or a chemically-induced depletion of the high energy phosphate stores mimics the effect of ischaemia on the density of the DHP binding sites in rat cardiac membane fragments. Chemically-induced high energy phosphate depletion was achieved by adding 2,4-dinitrophenol (DNP) (an uncoupler of oxidative phosphorylation) and iodoacetic acid (IAA) (an inhibitor of glycolysis) to the perfusion buffer.

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METHODS

Adult male Sprague–Dawley rats (250–300 g) were used for these studies. They were anaesthetized with diethylether–O_2 mixture and heparinized [6]. The hearts were then excised, arrested by immersion in ice-cold Krebs–Henseleit (K–H) buffer, trimmed, weighed and perfused with aerobic K–H buffer for 15 min at 37° in the Langendorff mode, using a mean coronary flow of 10–12 ml/min/g wet wt, as previously described [6]. The hearts were allowed to beat spontaneously.

Perfusion buffers

The K–H buffer (K–H) contained, in mM: NaCl, 119.0; KCl, 4.6; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.3 and glucose, 11.0. For aerobic perfusion it was gassed with 95% O_2 and 5% CO_2.

Glucose-free aerobic K–H buffer was prepared by substituting sucrose (22 mM) for glucose in the aerobic K–H.

Substrate-free hypoxic K–H was prepared as described for glucose-free aerobic K–H, but was gassed with 95% N_2 and 5% CO_2 instead of 95% O_2 and 5% CO_2.

Irrespective of their composition, the pH of the perfusion buffers was 7.3.

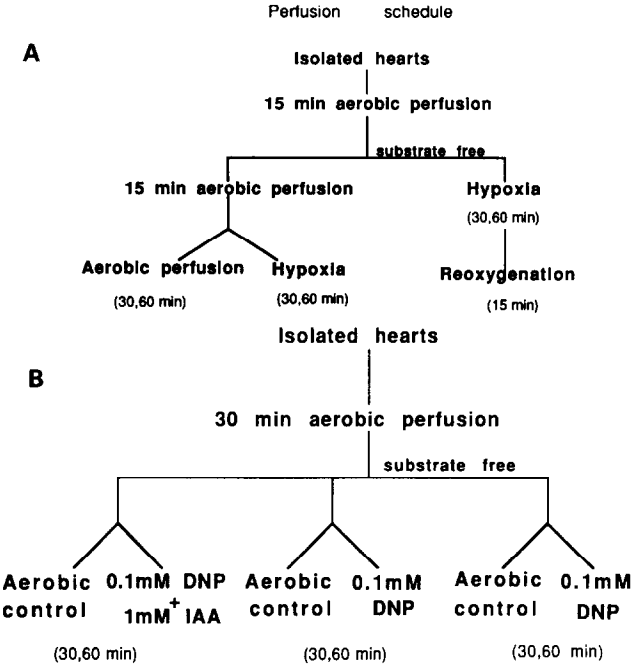

Fig. 1. Schematic representation of the perfusion schedules. The composition of the perfusion buffers is described in the text.

Perfusion schedule

Figure 1A and B shows the perfusion schedule adopted for these studies. Irrespective of the proposed perfusion sequence each heart was perfused initially with K–H for 15 min.

Aerobic series

These hearts were perfused with aerobic K–H for another 45 or 75 min, to provide the 30 and 60 min aerobic controls (30 min equilibration, plus 30 or 60 min K–H perfusion).

Hypoxia series

After the initial 15 min K–H perfusion, hearts in this series, (Fig. 1A) were randomly divided into 3 groups (i–iii).

(i) *Substrate-free aerobically perfused hearts.* These hearts were perfused for another 15 min with aerobic K–H and then for either 30 or 60 min with glucose-free aerobic K–H buffer (30 min equilibration, 30 or 60 min glucose-free aerobic perfusion).

(ii) *Substrate-free hypoxic hearts.* These hearts were perfused with aerobic K–H buffer for 15 min, and then made hypoxic, by perfusing with glucose-free hypoxic K–H for either 30 or 60 min.

(iii) *Reoxygenated hearts.* After either 30 or 60 min substrate-free hypoxic perfusion, reoxygenation was affected by perfusing with glucose-containing aerobic K–H for 15 min.

High energy phosphate (HEP) depletion series

(a) *HEP depletion due to uncoupling of oxidative phosphorylation.* Hearts in this series were perfused with aerobic K–H for 30 min before being randomly divided into two groups (Fig. 1B):

(i) *Substrate (glucose) present*

Hearts in this group were perfused for another 30 or 60 min with aerobic K–H buffer, in the presence or absence of 0.1 mM DNP. DNP was dissolved in warm K–H buffer.

(ii) *Substrate-free*

These hearts were perfused for 30 or 60 min as described above, but using glucose-free aerobic K–H, with and without 0.1 mM DNP.

(b) *HEP depletion due to inhibition of glycolysis and oxidative phosphorylation.* Hearts in this series (Fig. 1B) were perfused with aerobic K–H for 30 min and then divided into 2 groups.

(i) *Control hearts*

Here aerobic K–H perfusion was continued as described for another 30 or 60 min in the presence of glucose.

(ii) *DNP- and IAA-treated hearts*

Hearts in this group were perfused for either 30 or 60 min with aerobic glucose-containing K–H to which 0.1 mM DNP and 1 mM IAA had been added.

Throughout the experiments, care was taken to ensure that the left ventricular wall temperature was maintained at 37°. This involved surrounding the hearts with temperature-controlled water-filled jackets.

Measurement of 1,4-dihydropyridine (DHP) binding

Cardiac membrane isolation. At the end of the perfusion sequence, the hearts were immersed in ice-cold homogenizing medium containing 20 mM $NaHCO_3$, and 0.1 mM phenylmethylsulphonyl flouride (PMSF). Membranes were then isolated, by the method of Glossmann and Ferry [7]. The ventricles were trimmed of fat, minced into small pieces

Table 1. Protein yield of membrane fragments isolated from rat hearts perfused under different experimental conditions

Perfusion conditions	Protein yield (mg protein/g wet wt)	
	30-min series	60-min series
Hypoxic series		
Aerobic	8.46 ± 0.14	8.11 ± 0.12
Hypoxic	7.85 ± 0.15	8.40 ± 0.22
Reoxygenation	7.98 ± 0.13	8.31 ± 0.15
0.1 mM DNP series		
(a) *In the presence of glucose*		
Aerobic	8.12 ± 0.14	8.10 ± 0.16
0.1 mM DNP	8.26 ± 0.17	6.20 ± 0.10
(b) *In the absence of glucose*		
Aerobic	8.27 ± 0.12	8.17 ± 0.16
0.1 mM DNP	8.09 ± 0.13	8.23 ± 0.14
0.1 mM DNP and 1 mM IAA series		
Aerobic	8.85 ± 0.13	8.67 ± 0.21
0.1 mM DNP + 1 mM IAA	8.98 ± 0.22	9.12 ± 0.35

Each result is in the mean ± SE of 4–6 experiments.
DNP = 2,4-dinitrophenol.
IAA = iodoacetic acid.
Aerobic perfusion: glucose-containing K–H perfusion buffer gassed with 95% O_2 and 5% CO_2.
Hypoxic perfusion: glucose-free K–H perfusion buffer gassed with 95% N_2 and 5% CO_2.
Reoxygenation was 15 min, with glucose-containing aerobic K–H perfusion buffer.

(<1 mm^3), and homogenized in 8 ml homogenizing medium/g wet wt tissue, using an Ultra-Turrax homogenizer for 2 × 15 sec at ¾ maximum speed. The homogenate was diluted 1 : 20 (wet wt: homogenizing vol.) and then centrifuged at 1500 *g* for 15 min at 4°. The pellet was discarded, and the supernatant recentrifuged for 15 min at 48,000 *g*. The resultant pellet was suspended in ice-cold 50 mM Tris buffer containing 0.1 mM PMSF, (pH 7.4) and recentrifuged at 48,000 *g*. This procedure was repeated twice. The pellet from the final centrifugation step was suspended in 50 mM Tris buffer, to provide a final protein concentration of 0.8–1.2 mg/ml, and stored in liquid N_2 [8]. Protein was assayed as described by Lowry *et al.* [9], using bovine serum albumin as standard.

(+)[^{3}H]PN200-100 binding

1,4-dihydropyridine binding was monitored as described by Glossmann and Ferry [7], using (+)[^{3}H]PN200-110. Binding was performed in duplicate at 25°, using a protein concentration of 0.2–0.5 mg/ml, in a final assay volume of 0.25 ml. The incubation medium contained 50 mM Tris and 0.1 mM PMSF (pH 7.4). For saturation binding 0.01–1.0 nM (+)[^{3}H]PN200-110 was used. Non-specific binding was defined by the addition of 1 μM (−)Bay K8644 and was less than 10% of the total binding. After 60 min incubation, bound and free (+)[^{3}H]PN200-100 were separated by rapid vacuum filtration across Whatman GF/C filters, after diluting with 3.5 ml ice-cold 10 mM Tris buffer containing 6.6% polyethyleneglycol (PEG) 6000 (pH 7.4). The filters were washed twice with Tris–PEG buffer, and the radioactivity of the filters counted (40% efficiency) in a Filter Count Scintillant (Packard, IL, U.S.), using a Packard Tricarb spectrometer. Binding selectivity was characterized by using enantiomers of the dihydropyridines, (+)PN200-110 ($10^{-13} - 3 \times 10^{-8}$ M), (−)PN200-110 ($10^{-11} - 10^{-6}$ M), (−)Bay K8644 ($10^{-12} - 3 \times 10^{-7}$ M), (+)Bay K8644 ($10^{-11} - 10^{-6}$ M), or (−)D600 (gallopamil) ($10^{-10} - 10^{-5}$ M), and the alpha adrenoceptor antagonist, prazosin ($10^{-10} - 10^{-5}$ M), to displace bound (+)[^{3}H]PN200-110. D-*cis* diltiazem ($10^{-10} - 10^{-5}$ M) stimulation of (+)[^{3}H]PN200-110 binding was monitored at 37° [10]. Binding studies were carried out under a sodium lamp, because of the photolability of the dihydropyridines.

Data analysis

Initial estimates of equilibrium binding parameters (K_d and B_{max}) were obtained from Scatchard, Hill and Hofstee analysis, using the "EBDA" programme [11]. A file was produced and the data analysed with the aid of a weighted, non-linear, least-squares computer curve fitting programme [12] to obtain final estimates. K_d is defined as the concentration of ligand required to occupy 50% of binding sites, and B_{max} as the density of the sites. The data was subjected to analysis of variance followed by modified Student's *t*-test (with the Bonferroni adjustment of *t* statistic for multiple comparison [13]). The level of significance was taken at $P < 0.05$.

Reagents

(+)[^{3}H]PN200-110 (specific activity 71–83 Ci/mmol) was obtained from Amersham International (U.K.). (+)PN200-110 and (−)PN200-110, were obtained from Sandoz (Switzerland) and (+)Bay

K8644 and (−)Bay K8644 were provided as gifts from Bayer AG, (F.R.G.). (−)D600 was a gift from Knoll AG, (F.R.G.), and d-*cis* diltiazem from Tanabe Laboratories (Japan). Prazosin was obtained from Pfizer Ltd (Australia). IAA and all other reagents were from Sigma Chemical Company (St. Louis, MO). DNP was supplied by the Ajax Company (Sydney, Australia).

RESULTS

Characterization of cardiac membrane fragments

Table 1 shows that the yield of membrane obtained from the aerobically perfused hearts was not significantly different from that obtained from either hypoxic or reoxygenated hearts, or from hearts which had been perfused with either DNP or DNP plus IAA-containing K–H buffer, or glucose-free K–H buffer (Table 1). It is unlikely, therefore, that any change in K_d or B_{max} encountered in these studies can be attributed to the harvesting of different populations of membrane fragments.

(+)[^{3}H]PN200-110 binding to membranes isolated from non-perfused hearts

Membranes isolated from freshly excised hearts which had been immersed in, but not perfused with, K–H buffer, contained a single population of high affinity (+)[^{3}H]PN200-110 binding sites, with an affinity (K_d) of 0.044 ± 0.002 nM, a density (B_{max}) of 258.7 ± 18.3 fmol/mg protein, and a Hill coefficient which centred around unity.

The binding was saturable and stereoselective, with (+)PN200-100 > (−)Bay K8644 > (−)PN200-110 = (+)Bay K8644 in displacing bound (+)[^{3}H]PN200-110. (−)D600 allosterically displaced bound (+)[^{3}H]PN200-110, and d-*cis* diltiazem stimulated the binding. Prazosin had no effect.

Effect of aerobic perfusion with and without glucose substrate on (+)[^{3}H]PN200-110 binding

(i) *Hearts perfused with aerobic K–H buffer containing glucose.* Table 2 shows that membranes harvested from hearts which had been perfused with aerobic glucose-containing K–H for up to 90 min (30 min preliminary K–H perfusion followed by another 30 or 60 min K–H perfusion, Fig. 1A), retain a single population of high affinity (+)[^{3}H]PN200-110 binding sites, the K_d and B_{max} of which resemble those obtained for freshly excised, non-perfused hearts (see previous section).

(ii) *Hearts perfused with aerobic, glucose-free K–H buffer.* Table 2 shows that omitting glucose from the aerobic K–H failed to cause any change in the density (B_{max}) of these binding sites during 60 min perfusion. Under these same conditions of perfusion (glucose-free, aerobic K–H) the K_d was unchanged after 30 min, but longer periods of glucose-free perfusion (60 min, Table 2) caused an increase ($P < 0.05$) in the K_d ($K_d = 0.045 \pm 0.004$ nM) after 60 min perfusion with glucose-containing aerobic K–H, compared with 0.078 ± 0.005 nM after the same period of perfusion with glucose-free, aerobic K–H). Irrespective of the perfusion conditions, however, the Hill coefficients remained centred around unity (Table 2), and selectivity was maintained (Fig. 3A

Table 2. (+)[^{3}H]PN200-110 binding to membranes isolated from aerobically perfused, hypoxic and reoxygenated rat hearts

Perfusion conditions	K_d (nM)	B_{max} (fmol/mg protein)	Hill coefficient	K_d (nM)	B_{max} (fmol/mg protein)	Hill coefficient
	30-min series			60-min series		
Aerobic (with glucose)	0.042 ± 0.003	210.4 ± 7.3	0.980 ± 0.008	0.045 ± 0.004	219.3 ± 20.3	0.983 ± 0.008
Aerobic (without glucose)	0.044 ± 0.002	194.0 ± 15.7	0.973 ± 0.009	0.078* ± 0.005	215.7 ± 16.0	0.993 ± 0.004
Hypoxic (without glucose)	0.048 ± 0.002	181.7 ± 6.9	0.979 ± 0.007	0.080* ± 0.005	224.4 ± 5.8	0.998 ± 0.003
Reoxygenation (with aerobic K–H)	0.058‡ ± 0.002	169.8 ± 12.4	0.986 ± 0.005	0.077* ± 0.005	172.3†‡ ± 9.6	0.993 ± 0.004

* $P < 0.05$ relative to the appropriate group of 30 min series under the same conditions.
† $P < 0.05$ relative to the appropriate aerobic perfusion groups with glucose.
‡ $P < 0.05$ relative to the aerobic controls (without glucose) and hypoxia.
Each result is the mean ± SE of 4–6 separate experiments. Tests of significance were calculated at the $P < 0.05$ level of significance with the Bonferroni modification of the *t* statistic for multiple comparison. Reoxygenation was for 15 min with glucose-containing perfusion buffer gassed with 95% O_2 and 5% CO_2.

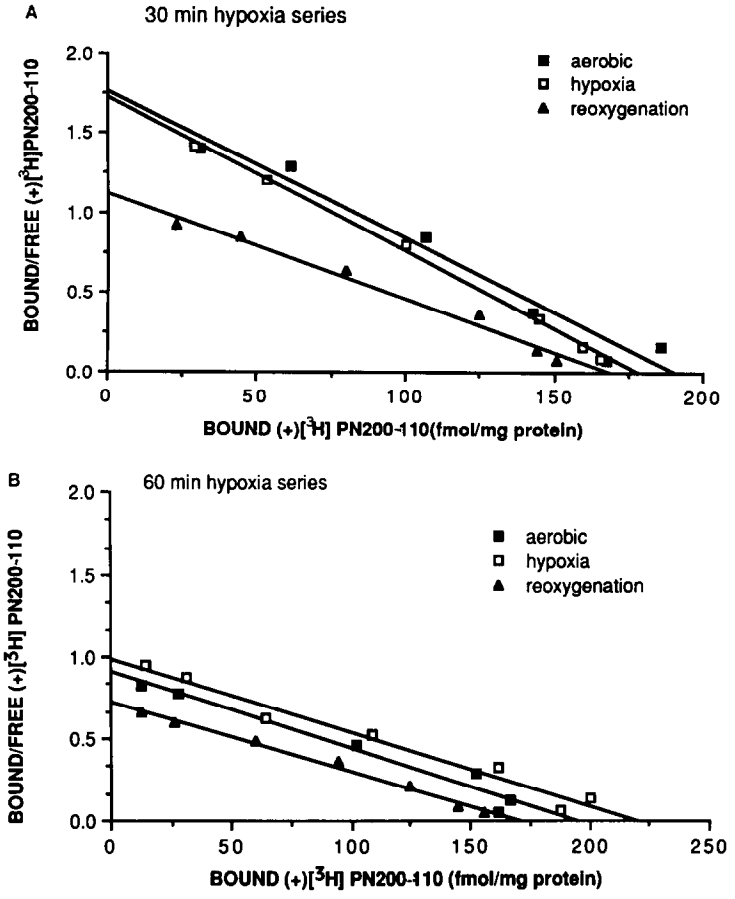

Fig. 2. Representative Scatchard plots of (+)[³H]PN200-110 binding to membrane fragments isolated from aerobically perfused (■), hypoxic (□) and reoxygenated (▲) isolated rat hearts. (A) refers to 30 min, and (B) to 60 min series (see text). Non-specific binding was less than 10% of total binding (see text). The binding data is presented in Table 2. Similar estimates were obtained from 6 separate experiments, using duplicate estimates for each point.

and D), with (+)PN200-110 > (−)Bay K8644 > (−)PN200-110 = (−)Bay K8644 ≫ (−)D600 in displacing (+)[³H]PN200-100. D-*cis* diltiazem continued to stimulate the binding, and prazosin had no effect.

In summary, therefore, the B_{max}, K_d and selectivity of the DHP binding sites in rat cardiac membranes survive aerobic perfusion, provided that the perfusion buffer contains glucose as substrate. In the absence of glucose substrate B_{max}, K_d and selectivity remain constant during 30 min perfusion, but within 60 min the affinity of these sites decreases (Table 2), although their density (Table 2) and selectivity (Fig. 3) are unchanged, and the Hill coefficients (Table 2) remain centred around unity.

Effect of hypoxic perfusion on (+)[³H]PN200-110 binding

Figure 2 and Table 2 show that neither 30 nor 60 min glucose-free hypoxia has any effect on the B_{max} of the DHP binding sites, relative to the values obtained after equivalent periods of glucose-free aerobic perfusion. As observed for the glucose-free aerobically perfused hearts, glucose-free hypoxic perfusion also caused a significant increase ($P < 0.05$) in the K_d of the DHP binding sites during 60, but not 30, min perfusion (Fig. 2A and B, Table 2). The selectivity of the sites was again maintained (Fig. 3B and E).

Hence, although the K_d of the (+)[³H]PN200-110 binding sites increases during 60 (but not 30) min glucose-free hypoxia, a similar increase occurs during 60 min perfusion with glucose-free aerobic K–H. By contrast, B_{max} remains constant during 60 min perfusion irrespective of whether perfusion is with glucose-free hypoxic or aerobic K–H. The selectivity is also unchanged.

Effect of reoxygenation on (+)[³H]PN200-110 binding

Membranes harvested from hearts which had been reoxygenated with glucose containing aerobic K–H for 15 min after 30 or 60 min hypoxia retained a single population of DHP binding sites (Fig. 2A and B). Table 2 shows that although reoxygenation after 30 min hypoxia failed to cause any significant change in the B_{max} of these binding sites, there was a small but significant increase in K_d. By contrast, reoxy-

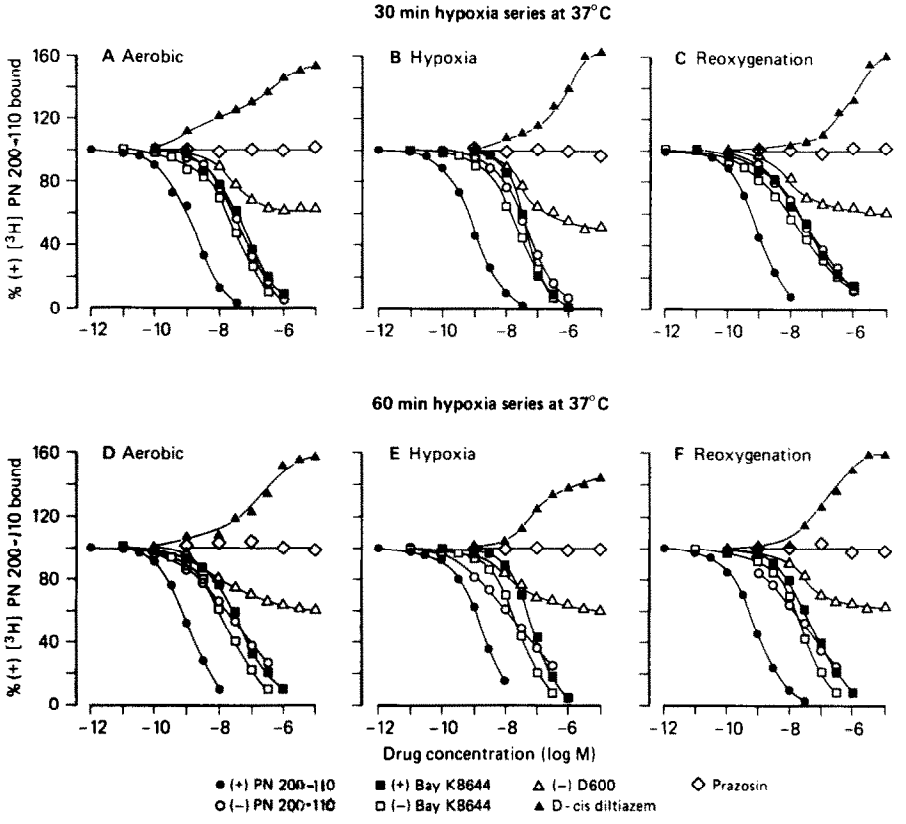

Fig. 3. Allosteric and stereoselective interaction of (+)PN200-110 (●), (−)PN200-110 (○), and (+)Bay K8644 (■), (−)Bay K8644 (□), (−)D600 (△), d-*cis* diltiazem (▲), and prazosin (◇), with (+)[^{3}H]PN200/110 binding sites in cardiac membranes isolated after glucose-free aerobic perfusion for 30 (A) or 60 (D) min, hypoxia for 30 (B) or 60 (E) min and after 15 min reoxygenation after 30 (C) or 60 (F) min of hypoxia. Each curve is the representative of 3 separate experiments.

genation after 60 min hypoxia resulted in a significant ($P < 0.05$) decrease in B_{max} relative to the B_{max} obtained after an equivalent period of either aerobic or hypoxic perfusion (Table 2). The K_d, however, was unaltered, and selectivity (Fig. 3C and F) was maintained, irrespective of whether reoxygenation was after 30 or 60 min hypoxia.

The effect of high energy phosphate depletion on (+)[^{3}H]PN200-100 binding

We, and others have previously established that 30 min perfusion with aerobic K–H containing 0.1 mM 2,4 DNP depletes the high energy phosphate [both adenosine triphosphate (ATP) and creatine phosphate (CP)] content of isolated rat hearts by more than 80% (Fig. 4 and Ref. 14). In the following section we shall consider the effect on DHP binding of high energy phosphate depletion as: (a) a result of uncoupling oxidative phosphorylation alone, or (b) together with inhibition of glycolysis.

(a) *High energy phosphate depletion due to uncoupling of oxidative phosphorylation with DNP.* (i) With glucose substrate present: In these experiments, the aerobic K–H contained glucose (see Methods). Figure 5 and Table 3 show that under these conditions 30 min perfusion with aerobic K–H containing 0.1 mM DNP caused a significant ($P < 0.05$) increase (48%) in the B_{max} of the DHP binding sites, without any change in affinity (K_d), compared to the relevant aerobic controls. Extending the DNP perfusion to 60 min caused the B_{max} to return to values close to those obtained after 60 min aerobic perfusion. Irrespective of the duration of the DNP perfusion (up to 60 min), the K_d and the selectivity of the binding sites were unchanged (Fig. 6A and B, and Table 3).

(ii) Without glucose substrate: As already described, although glucose-free aerobic perfusion has no effect on the density of the DHP binding sites (Tables 2 and 3, and Fig. 5), the K_d increases if the perfusion extends beyond 30 min. Table 3 shows that 30 min perfusion under these same conditions, but with 0.1 mM DNP present, causes a significant increase (27%, $P < 0.05$) in the B_{max}, again without any change in affinity or selectivity (Fig. 6C and D). Table 3 also shows that although 30 min glucose-free perfusion with 0.1 mM DNP increases the B_{max}, the increase does not persist during 60 min perfusion (Table 3 and Fig. 5).

Hence, irrespective of whether glucose is present as substrate, the B_{max} of the DHP binding sites increases during 30 min DNP perfusion, but the increase does not persist during 60 min perfusion. Irrespective of the duration of perfusion (30 or 60

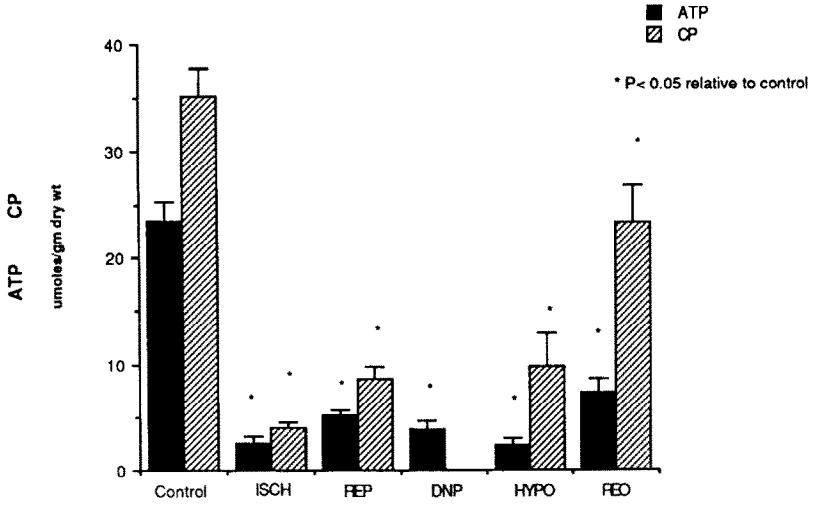

Fig. 4. Adenosine triphosphate (ATP) and creatine phosphate (CP) content of rat ventricular muscle after 60 min aerobic perfusion (control), 30 min ischaemia (ISCH), 30 min ischaemia followed by 15 min reperfusion (REP), 30 min perfusion with K–H containing 0.1 mM DNP (DNP), 30 min hypoxia (HYPO) and 30 min hypoxia followed by 15 min reoxygenation (REO) Experiments were performed at 37°. Each bar is mean ± SE of 6 separate experiments. (For methods see Refs. [6, 14]).

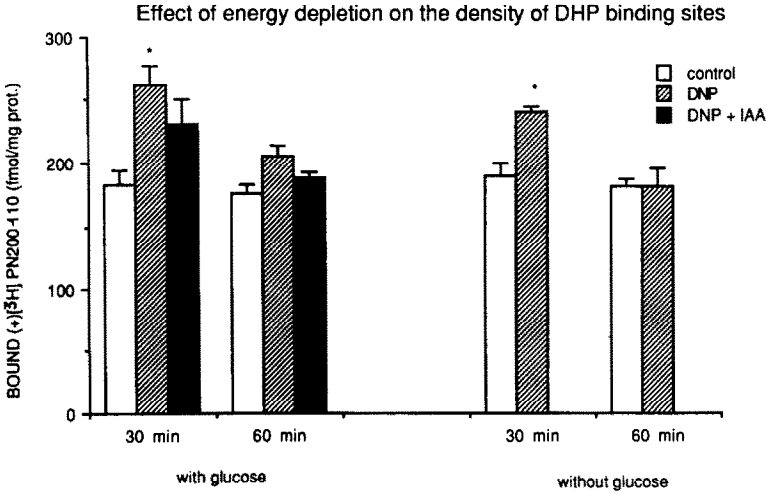

Fig. 5. Histogram of (+)[³H]PN200-110 binding site density (fmol/mg protein) monitored in cardiac membranes isolated from hearts that have perfused aerobically with K–H buffer (control) (□), with K–H buffer containing 0.1 mM DNP (▨), and with K–H buffer containing 0.1 mM DNP plus 1 mM IAA (■) for either 30 or 60 min at 37°. Estimates were obtained from 6 separate experiments.
* Is the significant difference in density of the DHP binding sites after perfusion with DNP containing K–H buffer relative to control at $P < 0.05$ level.

min) neither the affinity nor the selectivity of the binding sites was affected by DNP.

(b) *High energy phosphate depletion due to the combined inhibition of glycolysis and oxidative phosphorylation.* These experiments were undertaken to safeguard against the possibility of some ATP being produced via glycolysis, despite the presence of DNP. The hearts, therefore, were perfused with K–H containing DNP together with iodoacetic acid (IAA)—an inhibitor of glycolysis. Table 4 reveals that up to 60 min aerobic perfusion with 0.1 mM DNP and 1 mM IAA failed to change either the affinity or density of the DHP binding sites. The selectivity of the sites was also maintained, with (+)PN200-110 > (−)Bay K8644 > (−)PN200-110 = (+)Bay K8644 ≫ (−)D600 in displacing bound [³H]PN200-110, and d-*cis* diltiazem continuing to stimulate the binding.

The direct effect of DNP on DHP binding sites in cardiac membrane fragments

To exclude the possibility that DNP was having a direct effect on the DHP binding activity of these membranes, other experiments were undertaken in

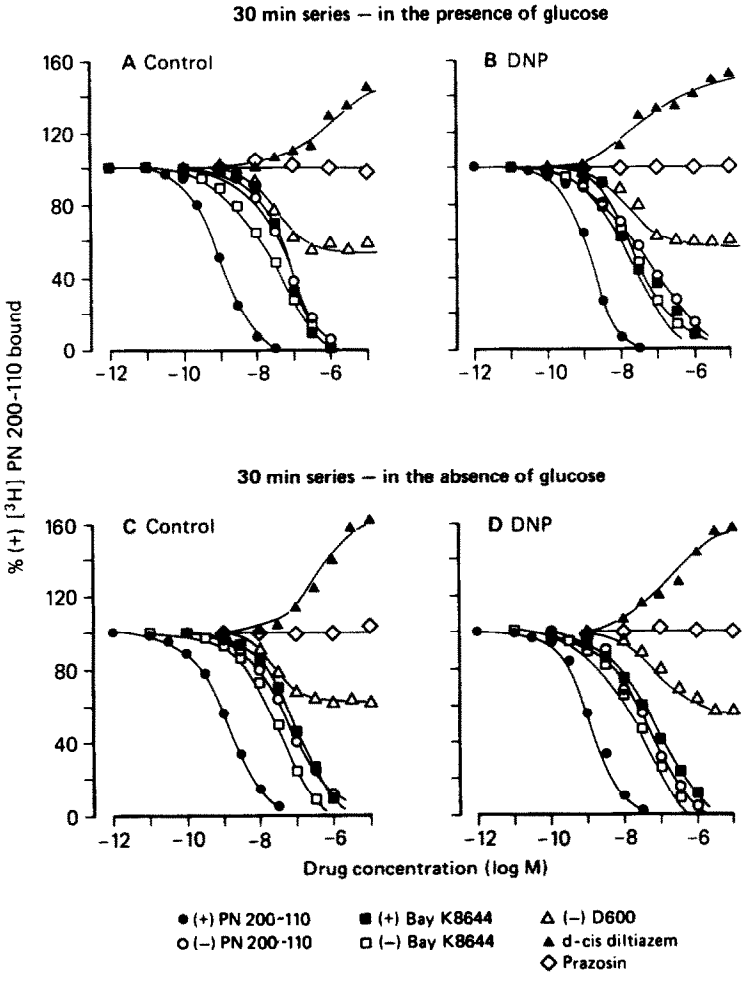

Fig. 6. Allosteric and stereoselective displacement of (+)PN200-110 (●), (−)PN200-110 (○), (+)Bay K8644 (■), (−)Bay K8644 (□), (−)D600 (△), d-*cis* diltiazem (▲), prazosin (◇) for (+)[^{3}H]PN200-100 bound to cardiac membranes isolated after aerobic perfusion for 30 min in the absence and presence of 0.1 mM DNP, with and without glucose substrate. Each curve is representative of 3 experiments.

which DNP was added directly to the incubation medium containing freshly harvested membrane fragments from non-perfused hearts. Two strategies were used. Either the effect of a single dose (0.1 mM) of DNP on the saturation binding of (+)[^{3}H]PN200-110 was determined, or displacement curves were established for increasing concentrations of DNP. These experiments were performed at two different temperatures—25°, the temperature at which binding studies were performed, and 37°, the temperature at which the hearts were perfused.

Saturation DHP binding experiments

Scatchard plots of (+)[^{3}H]PN200-110 binding to cardiac membrane fragments harvested from non-perfused hearts in the absence and presence of 0.1 mM DNP and incubated at 25° are shown in Fig. 7. Under these conditions, and even after 60 min incubation, DNP had no effect on either the K_d or B_{max} of the DHP binding sites.

Displacement experiments

The effect of $10^{-10} - 10^{-3}$ M DNP on the (+)[^{3}H]PN200-110 binding activity of freshly prepared membrane fragments when incubated at either 25° or 37° was studied. Over the concentration range used here DNP was without effect.

DISCUSSION

The present study shows that (+)[^{3}H]PN200-110 binds to high affinity dihydropyridine binding sites in rat cardiac membrane fragments, with an affinity (K_d) of 0.044 ± 0.002 nM, a density (B_{max}) of 258.7 ± 18.3 fmol/mg protein, and a Hill coefficient centering around unity. These values are similar to those obtained by other investigators [15, 16]. The study also shows that the DHP binding sites in rat cardiac membrane fragments survive prolonged periods of aerobic perfusion without any change in affinity, selectivity or density—provided that the

Table 3. (+)[^{3}H]PN200-110 binding to membranes isolated from rat hearts perfused under aerobic conditions, with and without 2,4-dinitrophenol (DNP)

Perfusion conditions	K_d (nM)	B_{max} (fmol/mg protein)	Hill coefficient	K_d (nM)	B_{max} (fmol/mg protein)	Hill coefficient
	In the presence of glucose			In the absence of glucose		
30 min series						
Aerobic	0.049 ± 0.005	186.0 ± 15.3	0.978 ± 0.008	0.048 ± 0.003	189.7 ± 10.5	0.982 ± 0.007
Aerobic plus 0.1 mM DNP	0.053 ± 0.004	275.0* ± 21.7	0.995 ± 0.003	0.048 ± 0.005	240.7* ± 4.0	0.987 ± 0.003
60 min series						
Aerobic	0.037 ± 0.002	175.5 ± 6.9	0.996 ± 0.010	0.058* ± 0.010	181.2 ± 5.9	0.982 ± 0.005
Aerobic plus 0.1 mM DNP	0.036 ± 0.001	205.7† ± 8.6	0.982 ± 0.007	0.055 ± 0.006	181.7† ± 14.3	0.980 ± 0.004

DNP = 2,4-dinitrophenol.
* $P < 0.05$ relative to the appropriate aerobic control.
† $P < 0.05$ relative to the 30 min group under same conditions.
Each result is mean ± SE of 6 separate experiments.
Tests of significance calculated at the $P < 0.05$ level, as described for Table 2.

Table 4. (+)[^{3}H]PN200-110 binding to membranes isolated from rat hearts perfused under aerobic conditions, with buffer containing DNP and IAA

Perfusion conditions	K_d (nM)	B_{max} (fmol/mg protein)	Hill coefficient	K_d (nM)	B_{max} (fmol/mg protein)	Hill coefficient
		30-min series			60-min series	
Aerobic	0.045 ± 0.003	182.3 ± 20.1	0.992 ± 0.004	0.034 ± 0.002	185.5 ± 10.1	0.969 ± 0.005
Aerobic plus 0.1 mM DNP + 1 mM IAA	0.037 ± 0.002	231.8 ± 19.7	0.991 ± 0.005	0.045 ± 0.002	189.0 ± 4.1	0.978 ± 0.004

DNP = 2,4-dinitrophenol.
IAA = iodoacetic acid.

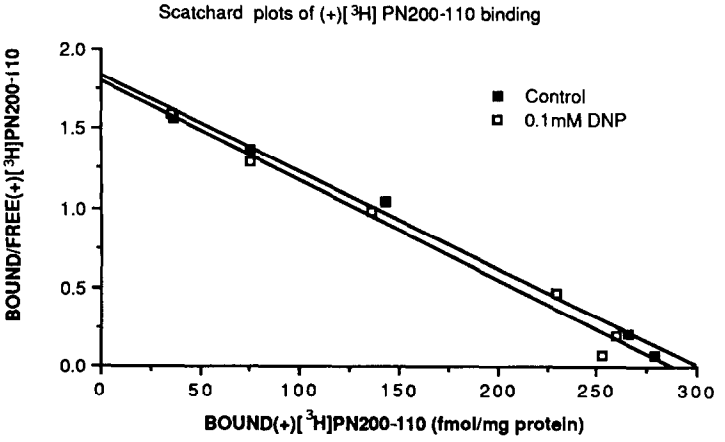

Fig. 7. Scatchard curves for (+)[^{3}H]PN200-100 binding to membranes harvested from freshly excised hearts. The membranes were incubated in reaction buffer without (control) (■) and with DNP (□) to provide a final concentration of 0.1 mM. Incubation was for 60 min at 25°. Non-specific binding was less than 10% of total binding (see text). Similar estimates were obtained for 6 separate experiments. ■ $K_d = 0.044 \pm 0.002$ nM; B_{max} 258.7 ± 18.3 fmol/mg protein. □ $K_d = 0.048 \pm 0.003$ nM; B_{max} 255.0 ± 15.6 fmol/mg protein.

perfusion buffer contains glucose, and that the sites exhibit a small but significant decline in affinity, without any change in density during 60 (but not 30) min of glucose-free aerobic perfusion.

Relative to the results obtained for membranes prepared from hearts which had been perfused for an equivalent period of time with glucose-free aerobic buffer, perfusion with glucose-free hypoxic buffer for up to 60 min failed to cause any further change in density, affinity or selectivity. These findings are in agreement with those described by Matucci *et al.* [17] for hypoxic guinea pig hearts. In addition, our results show that reoxygenation after 60, but not 30, min of glucose-free hypoxic perfusion, reduces the density of the binding sites, without altering their affinity or selectivity, and that perfusion with either glucose-containing or glucose-free aerobic buffer to which DNP or DNP plus IAA has been added has no effect on either the affinity or selectivity of these binding sites. Under these conditions of perfusion, however, the density of the DHP binding sites increased, relative to the aerobic controls, and provided that perfusion was for 30 and not 60 min.

In general, these results establish that neither hypoxic perfusion, nor chemically-induced, energy-rich, phosphate depletion mimics the effect of ischaemia on DHP binding. Previously we have established that ischaemia causes a time-dependent decrease in the density of these sites, without any change in affinity or selectivity [12]. These preliminary studies also established that under normothermic conditions (37°) this ischaemia-induced reduction in density (B_{max}) reaches asymptote within 30 min, that it is attenuated by hypothermic conditions (22°) and that it is reversed upon reperfusion after short (30 min), but not long (60 min) periods of ischaemia. The effect of ischaemia and of reperfusion on the binding activity of the DHP binding sites, therefore, differs markedly from that of hypoxia and reoxygenation—ischaemia causing a decrease and reperfusion an increase in B_{max}, whereas hypoxia has no effect beyond that caused by glucose-free aerobic perfusion. Similarly, chemically-induced energy depletion failed to mimic the effect of ischaemia, since it, like hypoxia, failed to reduce the density of these binding sites. Instead, 30 min of chemically-induced ATP depletion was associated with an increase in B_{max}. The effect of reoxygenation also differs from that of reperfusion, because whereas reperfusion can allow the B_{max} to return to control levels, reoxygenation either causes no change or a decrease.

Whilst the present results establish that neither oxygen-lack nor energy depletion can account for the ischaemia-induced reduction in the density of cardiac DHP binding sites, they fail to provide any explanation as to why ischaemia reduces the B_{max} of these sites. Other receptors respond differently. For example, the β_1-adrenoceptors are externalized during ischaemia [18, 19], resulting in an increase in B_{max}. However, acute episodes of hypoxia reduce the B_{max} of the β-receptors [20] by a mechanism which cannot be accounted for in terms of an altered rate of β-adrenoceptor synthesis [21]. The ischaemia-induced reduction in B_{max} of the DHP binding sites occurs rapidly [1, 2], and therefore probably cannot be accounted for in terms of a slowed rate of synthesis. Presumably it involves internalization of the sites. Alpha adrenoceptors resemble the β adrenoceptors in that they display an increase in B_{max} [22] in response to ischaemia.

According to our present results the ischaemia-induced reduction in the density of the DHP binding sites is mimicked neither by energy depletion nor O_2 lack. Other factors which are known to influence DHP binding site density include oestrogen—which increases the B_{max} in rat uterine smooth muscle cells [23]. Chemically-induced catecholamine depletion [24] also produces an increase in DHP binding site density, as does chronic ethanol injestion—at least in the brain [25]. According to our present results,

energy depletion due to the presence of metabolic inhibitors causes a biphasic change in binding site density, with an increase after 30, but no change after 60, min.

In conclusion, these results establish that the reduction in density of the cardiac DHP binding sites which occurs during ischaemia cannot be accounted for simply in terms of energy depletion or O_2-lack. Possibly a change in the phospholipid content of the sarcolemma is involved. Such an explanation could account for the decrease in B_{max} which occurs upon reoxygenation, since reoxygenation rather than hypoxia, is believed to be associated with massive perturbations of membrane structure and function [26].

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