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[^{3}H]-NITRENDIPINE BINDING IN MEMBRANES OBTAINED FROM HYPOXIC AND REOXYGENATED HEART

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Abstract—We compared the binding properties of [^{3}H]-nitrendipine in heart membranes from normal guinea-pig heart and from hypoxic or hypoxic and reoxygenated heart. The [^{3}H]-nitrendipine binds a single class of high capacity (B_{max} 667.2 ± 105.2) with high affinity (K_D 0.14 ± 0.02) binding sites. By contrast, in membranes of hypoxic and reoxygenated heart the B_{max} decreases significantly while it remains unaffected during hypoxia. Xanthinoxidase activity is increased in hypoxic-reoxygenated hearts.

Nifedipine, nitrendipine and related dihydropyridine compounds inhibit calcium-mediated phenomena in cardiac and vascular tissue. Contractile force studies and electrophysiological experiments have suggested that the mechanism of these compounds involves blockade of the voltage sensitive channels in sarcolemma [1, 2]. Binding sites for these ligands have been recently described in guinea-pig heart [3]. The present study investigates whether [^{3}H]-nitrendipine binding sites are changed in membranes obtained from normal, hypoxic and hypoxic-reoxygenated heart.

MATERIALS AND METHODS

Male guinea-pigs (250 g) were used. The heart was removed and perfused in a Langendorff apparatus. The hearts were initially equilibrated for 45 min with a solution containing (mM): NaCl 137, KCl 2.7, $MgCl_2$ 0.11, $CaCl_2$ 1.8, $NaHCO_3$ 12, NaH_2PO_4 0.42 and glucose 5 and gassed with O_2–CO_2 (97–3%).

Then they were perfused with a glucose-free medium gased with a mixture of N_2–CO_2 (97–3%) for 30 min (hypoxic phase).

Following the hypoxic phase, the hearts were reperfused with the original medium gassed with O_2–CO_2 (97–3%) for 30 min (reoxygenation phase). Details are described in Antonini *et al.* [4] and Franconi *et al.* [5].

After perfusion, the ventricle was dissected, cut into small pieces, bottled and weighed rapidly. Tissue was homogenized in 5 vol. (based on tissue weight) of 5 mM Tris–HCl buffer containing 250 mM sucrose pH 7.4, using an ultraturrax. The homogenate was filtered through one layer of cheesecloth and then centrifuged and processed as described in Williams and Lefkowitz [6]. Then the final suspension was taken for protein assay by Bradford's method [7] using bovine serum albumin as standard. Xanthinoxidase activity was measured spectrophotometrically in cardiac tissue as described in Rowe and Wyngaarden [8] with some modifications.

Kinetic binding experiments used 40 μg of protein, 1 nM [^{3}H]-nitrendipine 10^{-7} M cold nitrendipine, for 60 min incubation in Tris–HCl 50 mM buffer pH 7.5, 25°, to achieve a final volume of 125 μl.

The reaction was stopped by diluting 2 ml of buffer and rapidly filtering it on Whatman GF/B filters using a Millipore apparatus. The filter was washed twice with 5 ml of buffer, dried at 37° overnight and then placed in a scintillation cocktail containing Triton X-100 and counted in a TRICARB 460 C at an efficiency of 43%. Experiments in duplicate were systematically carried out and performed under sodium light because of the light sensitivity of dihydropyridine derivatives. The association kinetics of [^{3}H]-nitrendipine were determined in membranes obtained from normal, hypoxic and hypoxic-reoxygenated heart. Dissociation kinetics were determined by incubation membrane from normal hearts with 1 nM [^{3}H]-nitrendipine for 60 min, then adding 10^{-7} M unlabelled nitrendipine and interrupting the assay at various times.

Saturation experiments. These were performed in membranes obtained from normal and hypoxic-reoxygenated hearts. The normal conditions of binding were as follows 25 μg of protein, 0.02–0.5 nM [^{3}H]-nitrendipine; 10^{-7} cold nitrendipine, 60 min of incubation in 50 mM Tris HCl buffer pH 7.5, 25°, final volume 2 ml.

All data, unless otherwise specified, refer to specific bound, defined as excess tritium labelled [^{3}H]-nitrendipine bound over the non-specific binding definition (i.e. in the presence of 10^{-7} M unlabelled nitrendipine). Data from the saturation curve were analyzed by means of the Scatchard plot. The kinetic constant was calculated according to Weiland and Molinoff [9]. Statistical analysis was performed using Student's *t*-test with $P < 0.05$ taken as statistically significant.

Materials. [^{3}H]-nitrendipine (70 Ci·mmol, New England Nuclear, Boston, MA); nitrendipine and

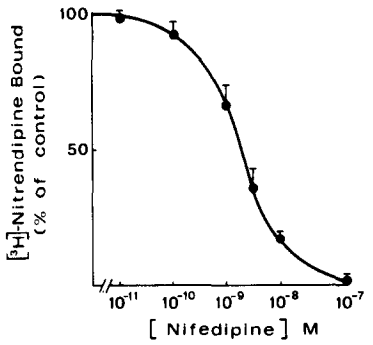

Fig. 1. Displacement of [^{3}H]-nitrendipine binding (1 nM) by increasing concentrations of nifedipine; each point was measured in duplicate at 25°. Values are means ± SE of four determinations.

0.5 nM [^{3}H]-nitrendipine in the presence and absence of 10^{-7} nitrendipine for 60 min (Fig. 3). The Scatchard analysis of saturation curves gave the following B_{max} 667.2 ± 105.2 fmole·mg^{-1} protein and K_D 0.14 ± 0.024 nM (Fig. 4, Table 1). After these an investigation was conducted as to whether hypoxic perfusion with a glucose free medium modified the time-course of [^{3}H]-nitrendipine binding, as shown in Fig. 5; the time-courses of [^{3}H]-nitrendipine binding with membranes from normal or hypoxic heart are practically the same. On the other hand, the binding of [^{3}H]-nitrendipine to membranes obtained from hearts perfused with hypoxic and glucose-free medium for 30 min and subsequently reoxygenated with a medium containing glucose, was statistically reduced (Fig. 5). This reduction was due to a decrease in the number of B_{max} as shown by the analysis of saturation curve (Fig. 4, Table 1).

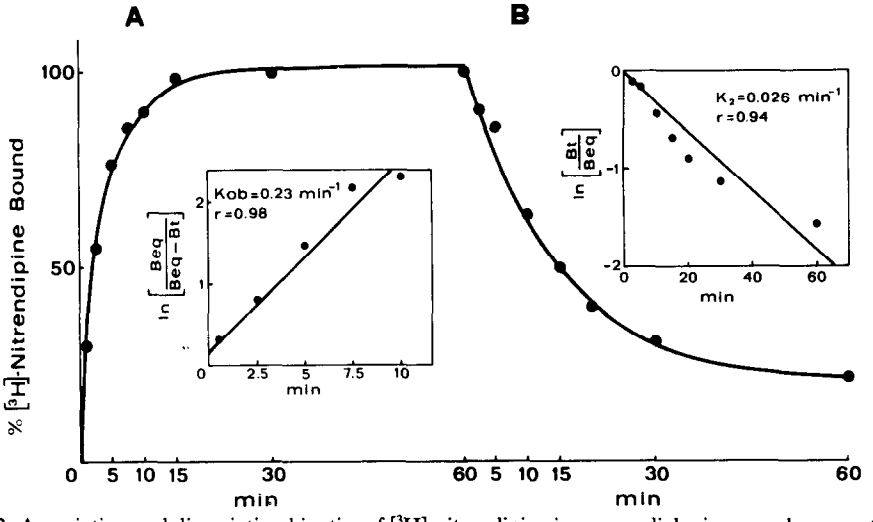

Fig. 2. Association and dissociation kinetics of [^{3}H]-nitrendipine in myocardial microsomal preparation. After equilibrium was reached and stable for more than 1 hr the rate of dissociation of the complex was monitored following the addition of 10^{-7} M cold nitrendipine. All experiments were performed in duplicate at 25° using 1 nM [^{3}H]-nitrendipine. Values of association are the means of six experiments, while values of dissociation are the means of three experiments. The insets in panels A and B show pseudo-first order rate of association (K_{ob}) and rate of dissociation (K_2) calculated according to Weiland and Molinoff [9].

nifedipine were a kind gift from Bayer AG, Leverkusen, F.R.G.; xanthinoxidase and xanthine (Sigma, St. Louis, MO). All other reagents were of analytical grade.

RESULTS

The binding was displaceable by nifedipine (Fig. 1). Saturable [^{3}H]-nitrendipine binding to the partially purified guinea-pig membranes increased with time. Using a [^{3}H]-nitrendipine concentration of 1 nM, the observed association rate constant (K_{ob}) is 0.23 min^{-1} (Fig. 2A); 10–15 min were required to reach steady-state binding at 25°. Addition of unlabelled nitrendipine to a final concentration of 10^{-7} M resulted in dissociation of [^{3}H]-nitrendipine with K_2 being 0.026 min^{-1} (Fig. 2B). The apparent dissociation constant derived from kinetic studies (K_2/K_1) was 0.14 nM. For saturation studies guinea-pig membranes were incubated with 0.02 nM to

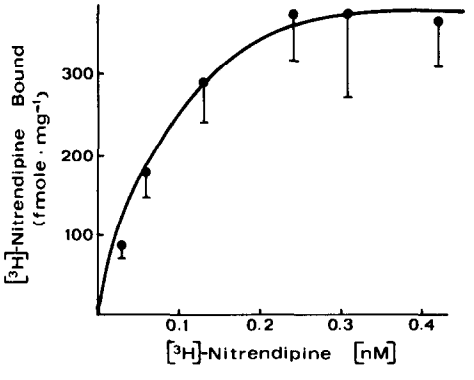

Fig. 3. Equilibrium specific binding of [^{3}H]-nitrendipine to guinea-pig microsomal membranes; each point was determined in duplicate and experiments were repeated four times. Values are means ± SE.

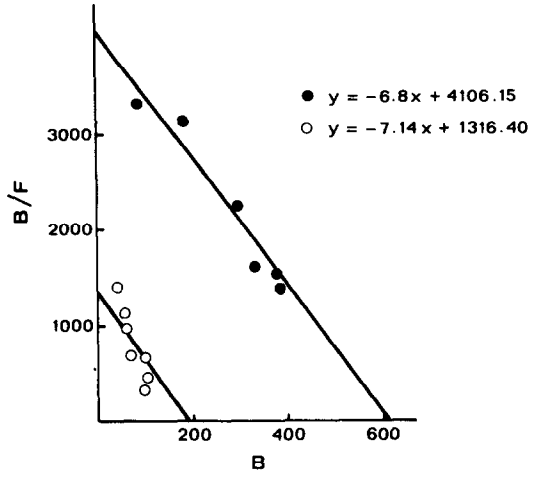

Fig. 4. Equilibrium binding data for [³H]-nitrendipine presented as Scatchard plot. Each data point is derived from the mean values of duplicate experiments. The concentrations of [³H]-nitrendipine varied between 0.02–0.5 nM. Protein concentration was 25 μg in a final volume of 2 ml. The open circles represent the reoxygenation experiments and the closed circles the control experiments.

The xanthinoxidase activity was increased statistically in the guinea-pig heart submitted to hypoxia followed by reoxygenation (Fig. 6).

DISCUSSION

In this study [³H]-nitrendipine binding characteristics are in general agreement with data obtained in other laboratories. The K_D calculated from the saturation curve of [³H]-nitrendipine was 0.14 ± 0.024 nM which is in the middle-range of values previously obtained in cardiac tissue [10, 11]. The number of [³H]-nitrendipine binding sites are also similar to that previously found [11]. The results of this study indicate that [³H]-nitrendipine binds rapidly and reversibly to a single population of high affinity sites. The similarity of the affinity constant of [³H]-nitrendipine with the equilibrium K_D value of radioligand derived from kinetic experiments suggests that the binding was measured under steady-state conditions and that the biochemical properties of radioactive and unlabelled nitrendipine are indistinguishable. It appears worthwhile to point out that after hypoxic phase the binding of [³H]-nitrendipine

Table 1. Effect of hypoxia (in the absence of substrate) and subsequent reoxygenation (in the presence of substrate) on [³H]-nitrendipine binding

	Control	Hypoxia (30 min) + Reoxygenation (30 min)
K_D (nM)	0.14 ± 0.024 (4)	0.12 ± 0.002 (5)
B_{max} (fmole·mg^{-1} protein)	667.2 ± 105.2 (4)	176.9 ± 19.4* (5)

Membranes are incubated as described in the text; values are means ± SE; in brackets the number of experiments.
* 0.05 > P > 0.01.

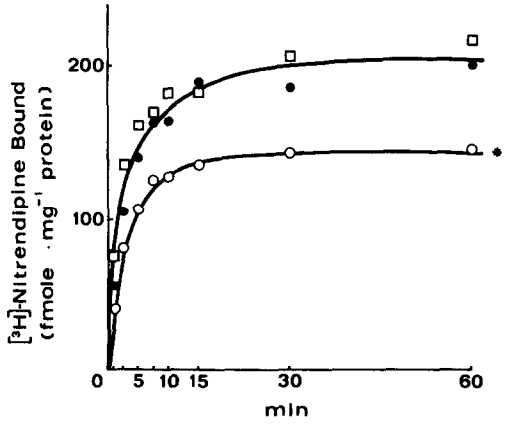

Fig. 5. Association curve of [³H]-nitrendipine to membranes of normal (●), hypoxic (□) and hypoxic and reoxygenated heart (○). Results are means ± SE of at least six experiments, each point being done in duplicate. * 0.05 > P > 0.01.

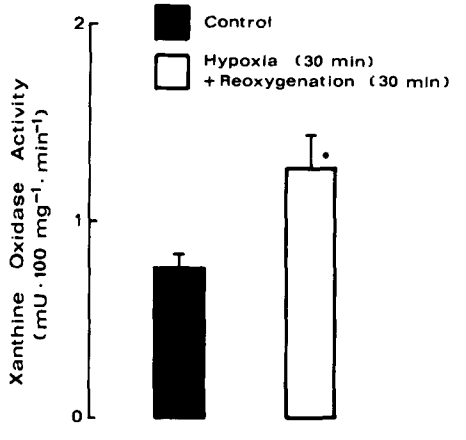

Fig. 6. Effect of hypoxia and reoxygenation on xanthinoxidase activity measured in cardiac tissue. The reaction mixture contained in a final volume of 3 ml: 0.1 M Tris–HCl buffer pH 8.1, 60 μM xanthine and 0.1 ml of cardiac supernatant. Formation of uric acid at 37° was measured at 292 nm at 1 min intervals. Values are means ± SE of six experiments. * 0.05 > P > 0.01.

is practically identical to the control situation; but reoxygenation, a situation that determines more marked damage at the cardiac level as tested by the increase in enzyme release and ventricular arrhythmias [5], determines a statistical decrease in [^{3}H]-nitrendipine binding. While this manuscript was in preparation Nayler *et al.* [12] described that [^{3}H]-nitrendipine binding was reduced during ischemia.

The formation of free radicals has been postulated during reoxygenation [13]. This is also suggested by the increase in xanthinoxidase activity which can by determined by ischemia and reperfusion [14]. It is not possible to conclude whether the reduction in the B_{max} is due directly to free radicals or whether it depends on other cellular damage. In fact, phospholipase C and neuraminidase decrease [^{3}H]-nitrendipine binding [15]. Therefore this binding appears to be affected by perturbation in phospholipids and sialic content of membranes; progressive degradation of sarcolemma phospholipids may be involved in the uncontrolled influx of Ca^{2+} from extracellular source into the cytoplasm [16, 17]. Moreover the increase of calcium influx could activate phospholipase and calcium activated protease [18, 19]; a release of this hydrolytic system might be involved in the generation of cellular damage.

However, in partially purified membranes obtained from isolated guinea-pig heart submitted to hypoxia in the absence of substrate followed by reoxygenation, the [^{3}H]-nitrendipine binding is reduced. Although the mechanism of this is not yet clear, it is possible that free radicals may play a role.

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