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[^{3}H]NIMODIPINE SPECIFIC BINDING TO CARDIAC MYOCYTES AND SUBCELLULAR FRACTIONS

A. DePover*, S.W. Lee**, M.A. Matlib, K. Whitmer, B.A. Davis, T. Powell†, and A. Schwartz§

Department of Pharmacology and Cell Biophysics
University of Cincinnati College of Medicine
Cincinnati, OH 45267 U.S.A.

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SUMMARY: [^{3}H]Nimodipine binding was studied in isolated myocytes from rat heart and in partially purified sarcolemma, sarcoplasmic reticulum and mitochondrial fractions from dog heart. In isolated myocytes, the density of [^{3}H]nimodipine specific sites (10^6 per cell) was close to the density of [^{3}H]QNB sites (0.8×10^6 per cell) and higher than that of [^{3}H]DHA sites (0.2×10^6 per cell). During subcellular fractionation, [^{3}H]nimodipine binding did not copurify with plasma membrane markers. The highest densities were found in fractions enriched in sarcolemma or in sarcoplasmic reticulum. No specific binding was found in mitochondria. These results indicate that the localization of [^{3}H]nimodipine sites is not restricted to areas of the plasma membrane rich in β-adrenoceptors, muscarinic receptors and sodium pump sites.

INTRODUCTION: High affinity sites for [^{3}H]nitrendipine and [^{3}H]nimodipine have been described in several tissues and it was proposed that these sites might be associated with calcium channels (1-13). According to divergent reports, [^{3}H]nitrendipine may or may not copurify with sarcolemmal markers (6,14,15). Furthermore, we have reported that the density of [^{3}H]nitrendipine sites was substantially lower than the density of [^{3}H]QNB sites (muscarinic receptors) and [^{3}H]DHA sites (β-adrenoceptors) in cardiac sarcolemma, but not in coronary artery microsomes (3). In order to clarify this important issue of localization, we have studied binding of [^{3}H]nimodipine to cardiac myocytes and to fractions enriched in mitochondria, sarcolemma and sarcoplasmic reticulum.

*Present address: Ciba-Geigy A.G., 141 Zlybeckstrasse, CH-4002 Basel, Switzerland.
**Visiting Scientist from College of Pharmacy, Yeungnam University, Gyongsan, Korea.
†University Laboratory of Physiology, Parks Road, Oxford, OX 13 PT, England.
§To whom reprint requests should be sent.
Abbreviations: QNB, quinuclidinyl benzilate; DHA, dihydroalprenolol

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METHODS: Isolation of myocytes from rat heart. Myocytes were prepared by enzymatic digestion of the ventricles as described by Powell et al. (16). Most cells were rod-shaped and striated, about 20 to 30% were damaged or leaky round cells. [^{3}H]Nimodipine binding to these preparations was not different from the binding studied to fractions containing 90% or more round cells. However, all data reported here were obtained with fractions enriched in rod cells. In some experiments, the cells were allowed to settle for 15 min and washed three times with physiological solution at room temperature in order to remove the albumin used during the isolation procedure.

Isolation of subcellular fractions from dog heart. Mitochondria, sarcolemma and sarcoplasmic reticulum were obtained as described respectively by Sordahl et al. (17), Van Alstyne et al. (18), and Kranias et al. (19). For the sake of clarity, the procedures are summarized in Fig. 1. All fractions were obtained from the same heart; sarcolemma and sarcoplasmic reticulum from the same homogenate. About 5 g of tissue was used for mitochondria (yield: about 10 mg/g tissue), and 180 g of tissue for sarcolemma (yield: 0.095 mg/g) and sarcoplasmic reticulum (yield: 0.40 mg/g). Medium I contained 180 mM KCl, 10 mM EGTA and 0.5% bovine serum albumin (pH 7.4); medium II, 0.3 M sucrose and 30 mM Tris-maleate (pH 7.0); medium III, 5 mM NaN_3 and 10 mM $NaHCO_3$ (pH 7.0); medium IV, 10 mM Tris-HCl (pH 7.4); medium V, 0.3 M sucrose, 0.6 M KCl and 20 mM Tris-maleate (pH 7.0); and medium VI, 0.3 M sucrose, 0.1 M KCl and 20 mM Tris-maleate (pH 7.0). Mitochondria were washed twice in 180 mM KCl to remove EGTA. All fractions were frozen at -30°C, thawed the next day and diluted to 1 mg/ml prior to use.

Binding and enzyme assays. Assays were carried out as reported elsewhere: binding of [^{3}H]ouabain, [^{3}H]DHA and [^{3}H]QNB (3), ATPase activities (20) and ^{45}Ca uptake (21). [^{3}H]Nimodipine (160 Ci/mmol) binding was estimated as described for [^{3}H]nitrendipine (3) except that the incubation volume was 0.575 ml for myocytes and 1 ml for subcellular fractions. One mM calcium was added to the incubation medium for binding assay of mitochondria, since it has been reported that EGTA inhibits [^{3}H]nitrendipine binding and calcium reverses this effect (8).

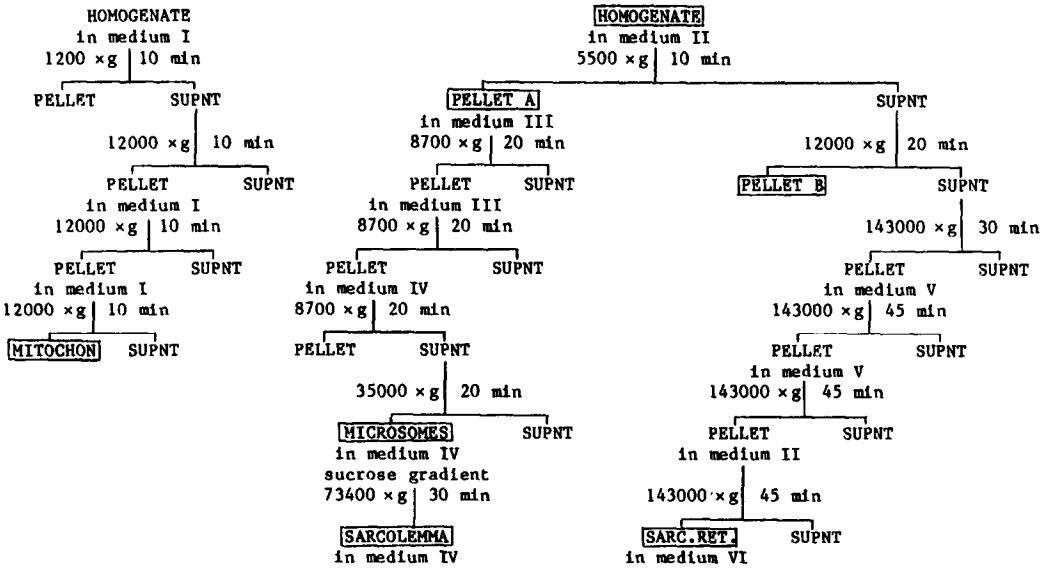

Figure 1. Flow diagrams of the procedures for isolating subcellular fractions from dog heart. Fractions enclosed by boxes in the figure were used for binding and enzyme assays (see Table 1). "SUPNT" refers to supernatants; "g" refers to maximal force.

RESULTS AND DISCUSSION: High affinity binding sites for [^{3}H]nimodipine have been reported in microsomal (9) and sarcolemmal preparations (10) respectively from rat heart (K_D = 0.24 nM) and dog heart (K_D = 0.18 nM). Fig. 2 shows [^{3}H]nimodipine binding to washed and unwashed isolated rat heart myocytes. The cells were washed in order to remove the albumin used in the preparation procedure. Extensive washing, however, was avoided to prevent cell damage. The binding was considerably higher in washed than in unwashed cells. In the washed cells (Fig. 3), the estimated K_D was 1.07 nM and B_{max}, 1.6 pmol per 10^6 cells or 10^6 sites per cell (see Footnote). For a protein content of 5.7

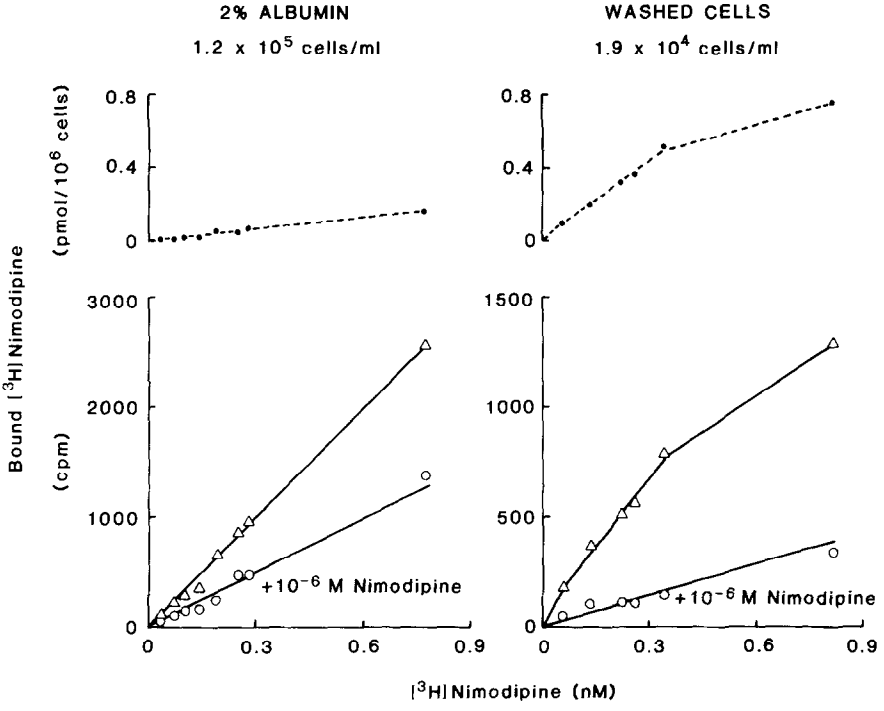

Figure 2. Effect of albumin wash-out on [^{3}H]nimodipine binding to rat heart myocytes. Suspensions of myocytes were equilibrated in nominally Ca-free Krebs-Ringer bicarbonate buffer (16) for 30 min at 30°C with various concentrations of [^{3}H]nimodipine and in the absence (Δ) or in the presence (o) of 10^{-6} M nimodipine. Left panels: unwashed cells (1.2×10^5 cells per ml). Right panels: washed cells (1.9×10^4 cells per ml). Lower panels: total (Δ) and non-specific binding (o). Upper panels: specific binding (specific = total - non-specific). The data represent one typical experiment. In separate experiments, association and dissociation of specific binding were carried out. Steady-state was reached within 10 min at 37°C. Dissociation at 37°C (chase with excess unlabelled nimodipine) was also complete within 10 min.

FOOTNOTE: For comparison, in electrophysiological studies, Reuter has estimated the number of active voltage-sensitive calcium channels to be from 2,000 to 10,000 per rat cardiac cell (25).

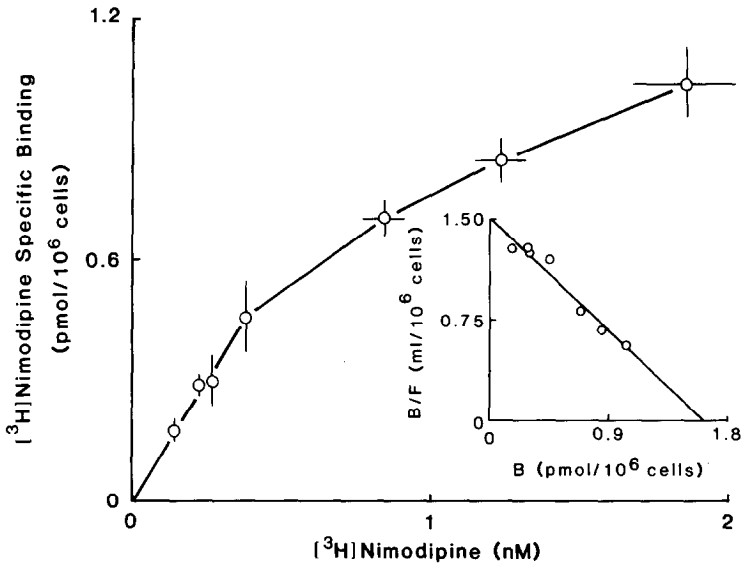

Figure 3. [^{3}H]Nimodipine specific binding to isolated myocytes (washed cells). The data represent means (± S.E.M.) from experiments with 4 different myocytes preparations (1-2 × 10^4 cells per ml). Same conditions as described in the legend to Fig. 2. Inset: Scatchard plot.

ng per cell (22), the calculated B_{max} was 284 fmol/mg. The [^{3}H]nimodipine concentrations used in these experiments did not exceed 2 nM, above which non-specific binding rose sharply. Thus, it was not possible to study a large range of concentrations in order to assess for the possible presence of more than one binding site. Similar experiments carried out with [^{3}H]QNB and [^{3}H]DHA gave B_{max} values respectively of 0.027 and 2.2 nM, and B_{max} values of 0.8 × 10^6 and 0.2 × 10^6 sites per cell. The [^{3}H]DHA binding data are in good agreement with a previous report (23). For [^{3}H]QNB and [^{3}H]DHA, K_D values in myocytes were identical to the values obtained in a rat sarcolemma preparation (data not shown). The relatively high K_D for [^{3}H]nimodipine in the isolated myocytes is an apparent value probably due to albumin adhering to the cells.

The distribution of [^{3}H]nimodipine binding was compared to that of various membrane markers in subcellular fractions from dog heart (Table 1). The highest enrichment of [^{3}H]nimodipine binding was found in the fraction highly enriched in plasma membrane markers ([^{3}H]QNB, [^{3}H]DHA, [^{3}H]ouabain and Na,K-ATPase). This fraction was referred to as "sarcolemma." However, with purification factors of only 10 from the homogenate and 2 from the

Table 1. [^{3}H]Nimodipine specific binding to subcellular fractions from dog heart. 50 µg protein was incubated for 15 min at 37°C in 1 ml medium containing 0.7 nM [^{3}H]nimodipine, 50 mM Tris-HCl (pH 7.4) and in the presence or in the absence of 10^{-5} M diltiazem (± DTZ). Azide-sensitive ATPase (NaN_3), Na,K-ATPase (Na-K), Ca-ATPase (Ca), ATP-dependent oxalate-supported ^{45}Ca uptake, [^{3}H]ouabain binding (at 100 nM of label), [^{3}H]DHA binding (at 10 nM) and [^{3}H]QNB binding (at 0.2 nM) were also assayed. [^{3}H]Nimodipine, [^{3}H]DHA and [^{3}H]QNB concentrations were 3- to 5-fold above K_D, hence the binding data do not represent B_{max}. The data represent means from three preparations (± S.E.M.). In six different preparations, [^{3}H]nimodipine binding to "sarcolemma" and "sarcoplasmic reticulum" was respectively 630 ± 140 fmol/mg and 190 ± 33 fmol/mg.

	ATPase ACTIVITIES (µmol ADP·mg^{-1}·h^{-1})			ATP and OXALATE DEPENDENT ^{45}Ca UPTAKE (nmol·mg^{-1})	RADIOLIGAND BINDING (fmol·mg^{-1})				
	NaN_3	Na-K	Ca		OUAB	DHA	QNB	NIMODIPINE -DTZ	NIMODIPINE +DTZ
HOMOGENATE	35 (7)	3.0 (0.7)	3.6 (1.2)	22 (4)	2400 (200)	19 (8)	79 (30)	30 (15)	34 (16)
PELLET A	36 (6)	4.1 (1.3)	5.4 (1.1)	35 (11)	2800 (400)	21 (3)	77 (11)	28 (3)	27 (1)
PELLET B	72 (21)	7.4 (0.7)	13.5 (0.9)	139 (31)	5900 (300)	60 (48)	197 (44)	85 (20)	197 (70)
MITOCHONDRIA	56 (12)	2.2 (0.8)	0.5 (0.5)	0	2400 (700)	13 (7)	43 (23)	19 (9)	19 (6)
MICROSOMES	33 (9)	10.2 (0.9)	14 (2)	497 (147)	30300 (4800)	325 (74)	1259 (97)	224 (32)	415 (50)
SARCOLEMMA	0	97 (12)	10 (7)	135 (55)	185000 (33000)	1924 (746)	7911 (844)	379 (86)	657 (162)
SARCOPLASMIC RETICULUM	4.5 (3.6)	4.1 (3.5)	83 (15)	1990 (88)	27300 (2100)	213 (54)	1511 (381)	236 (41)	450 (63)

"microsomes," [^{3}H]nimodipine binding clearly did not copurify with plasma membrane markers. Consistently, binding was also found in the fraction enriched with sarcoplasmic reticulum markers (Ca-ATPase and ATP-dependent oxalate-stimulated ^{45}Ca uptake). This fraction accounted for 15% of the total sites present in the homogenate. The yield was only 6% for the sarcolemmal fraction. The low yield of "purified" fractions might account for discrepancies in data between laboratories (5-7,14,15). [^{3}H]Nimodipine binding was comparatively lower in pellet B, which was enriched in mitochondrial azide-sensitive ATPase, and was very low in highly purified mitochondria. The K_D for [^{3}H]nimodipine in sarcoplasmic reticulum (0.25 ± 0.11 nM, N=4) was not different from the value previously reported for sarcolemma (3). Diltiazem-

induced stimulation of the binding reported by us (3) and others (6,13) was not different in the two fractions (Table 1).

In conclusion, the density of [^{3}H]nimodipine sites in cardiac myocytes is in the same order of magnitude or higher than that of muscarinic receptors or β-adrenoceptors. However, localization is not restricted to sarcolemmal areas which have the highest density of plasma membrane markers. This is consistent with the preliminary conclusions of Williams and Jones (15) and seems to be the case for skeletal muscle (5,7). It is possible that the binding sites are located in T-tubules, junctional sarcoplasmic reticulum, interior and peripheral coupling with transverse tubules and surface sarcolemma and junctional "feet" processes, which are present in cardiac sarcoplasmic reticulum preparations (24).

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