

## Heterogeneity of Calcium Channel Agonist Binding Sites in the Coronary Artery

J. DŘÍMAL

*Institute of Experimental Pharmacology, Centre of Physiological Sciences, Slovak Academy of Sciences, Dúbravská cesta 9, 842 16 Bratislava, Czechoslovakia*

**Abstract.** The binding properties ( $^3\text{H}$ ) BAY k 8644 a 1,4-dihydropyridine calcium channel agonist were studied in the subcellular membrane fraction isolated from the coronary artery by differential centrifugation. The specific binding of ( $^3\text{H}$ ) BAY k 8644 to microsomal membranes of the coronary smooth muscle was rapid, saturable, reversible and of both high and low affinity. The dissociation constants obtained from Scatchard analysis with ( $^3\text{H}$ ) BAY k 8644 and nitrendipine were  $0.60 \pm 0.02 \text{ nmol.l}^{-1}$  and  $9.1 \pm 0.1 \text{ nmol.l}^{-1}$  for the high and low affinity binding site respectively and the estimated maximal numbers of binding sites in the plasma membrane fraction were  $0.76 \pm 0.02$  and  $3.15 \pm 0.18 \text{ pmol.mg}^{-1}$  of protein respectively. The substituted dihydropyridine calcium channel antagonists nitrendipine and nifedipine competitively inhibited specific ( $^3\text{H}$ )BAY k 8644 binding suggesting a common high affinity 1,4-dihydropyridine binding site in the coronary microsomal fraction for calcium channel activator and antagonists. The low affinity agonist binding sites were significantly inhibited by adding nucleoside carrier inhibitors, 2-deoxyadenosine and dipyridamole, and by -SH alkylating agent N-ethylmaleimide. The results suggests that the coronary artery contains both high and low affinity calcium channel binding sites (in a 1:5 ratio) with the low affinity calcium channel agonist binding sites being associated with nucleoside carrier and/or with-SH groups.

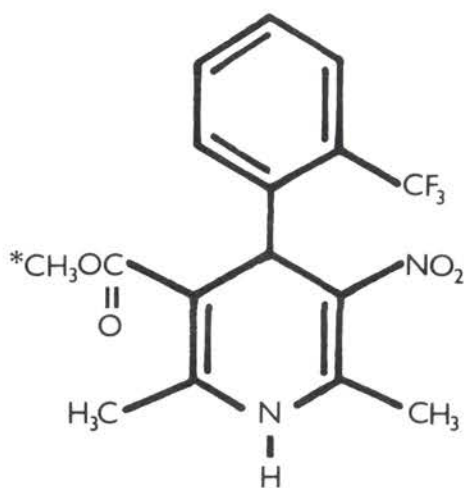
**Key words:** Calcium channels — Coronary artery — Vascular smooth muscle plasma membrane — Ligand binding — ( $^3\text{H}$ ) BAY k 8644-1,4-dihydropyridine calcium channel agonist — Nitrendipine

### Introduction

Vascular smooth muscle is activated through calcium release from intracellular calcium stores (van Breemen et al. 1972) and through transmembrane calcium

fluxes via specific proteinaceous calcium channels (Fleckenstein 1977). The most potent calcium channel antagonists, 1,4-dihydropyridines (1,4-DHP) inhibit the movement of calcium through calcium channels in excitable cells. BAY k 8644 (1,4-dihydro-2,6 dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylic acid methylester (Fig. 1) is a new 1,4-DHP derivative differing in its pharmacological action from other 1,4-DHP. BAY k 8644 acts directly on calcium channels in heart muscle to increase calcium current (Hess et al. 1984). It surprisingly constricts coronary resistance vessels at low, and reduces the coronary vascular resistance at high concentrations (Nyborg 1984; Dube et al. 1985). In porcine coronary artery rings nitrendipine pretreatment caused paradoxical potentiation of BAY k 8644 induced constriction (Grupp et al. 1984). It has been proposed that both excitatory and inhibitory 1,4-DHP receptors may be inclosed in the coronary artery and that the receptor subtypes may participate on the mediation of contraction or relaxation in coronary vascular smooth muscle (Dube et al. 1985).

Recent developments in technique of isolation of vascular smooth muscle cell membranes (Kwam et al. 1983), the possibility of obtaining large quantities of



BAY k 8644

Fig. 1. Structural formula of the calcium channel activator BAY k 8644. Asterisk indicator: The position of the tritium atom in the radiolabelled compound.

receptors for receptor binding assays by radioligand methods as well as the introduction of ( $^3\text{H}$ )BAY k 8644 at a reasonable specific activity have provided the opportunity to study calcium channel 1,4-DHP receptors also in the coronary artery.

The present paper reports on characteristics of ( $^3\text{H}$ )BAY k 8644 binding sites in the plasma membrane fractions of the coronary artery smooth muscle.

## Materials and Methods

Young dogs (8–10 kg) were anesthetized with sodium pentobarbital, artificially respired, and the hearts were quickly removed. Segments of the circumflex coronary artery (proximal 40 mm) were dissected free of surrounding myocardial, fat or connective tissue, cut into small pieces and homogenized in nine volumes of ice cold  $0.35 \text{ mol.l}^{-1}$  sucrose and  $5 \text{ mmol.l}^{-1}$  Tris-HCl (pH = 7.51). The preparations were treated with eight 10 s bursts each in an Ultraturrax homogenizer at 3/4 speed setting with 1 min cooling periods between the bursts.

### *Preparation of the Membrane Fraction*

Homogenates of the coronary artery were pressed under vacuum through stainless steel wire mesh and centrifuged at  $1500 \times g$  for 10 minutes at  $4^\circ\text{C}$ . The supernatant was centrifuged further at  $100,000 \times g$  for 10 minutes at  $4^\circ\text{C}$  in an M. O. M. ultracentrifuge. The resulting pellet was resuspended in assay buffer ( $50 \text{ mmol.l}^{-1}$  Tris-HCl, pH = 7.51) in an Elvehjem homogenizer with three gentle strokes of a Teflon pestle.

### *Binding Assays*

For the binding assays,  $100 \mu\text{l}$  aliquots of membrane fraction ( $0.065$ – $0.150 \text{ mg}$  protein) were added to the Eppendorf tubes containing assay buffer. Membrane fraction protein was incubated under standard assay conditions ( $25^\circ\text{C}$ ) with low concentrations of ( $^3\text{H}$ )BAY k 8644 ( $0.1$ – $3.0 \text{ nmol.l}^{-1}$ ) for 60 minutes. After the incubation, the assay mixture was diluted with 1 ml of  $4^\circ\text{C}$  buffer, immediately filtered through a Whatman GFB Glass Microfibre Filter and washed with 4.5 ml portions of the same buffer. Radioactivity collected on each filter was determined by liquid scintillation spectrometry in 10 ml SLD Scintillation liquid in a Packard 3-Carb Model 300C (Downs Grove, IL, USA) at an efficiency of 40 %. Specific binding of the radioligand was taken as the difference between total binding of the radioligand and that in the presence of  $10 \mu\text{mol.l}^{-1}$  nitrendipine or  $10 \mu\text{mol.l}^{-1}$  BAY k 8644. For all the assays involving small amounts of protein a single manifold filtration was carried out using Microsample Filtration Manifold with controllable flow rate. The purity of the radioactive ligand used in this study was routinely examined by thin layer chromatography on silica gel plates according to recommendations of the manufacturer. The binding of ( $^3\text{H}$ )BAY k 8644 (NEN Res. Product, DuPont, specific activity  $86 \text{ Ci mmol}^{-1}$ ) to glass fiber filters were minimized using filters presoaked with assay buffer. The binding assays were performed in triplicate and all procedures were carried out strictly under sodium light. Inter-group differences were analyzed statistically by Student's *t*-test. Scatchard analysis of binding, and the affinity spectra method (Tobler and Engel 1983) were used to calculate heterogeneity of binding sites. The preincubations included storing of fresh plasma membrane preparations with respective modifying agent for 30 min at  $4^\circ\text{C}$ , and the procedure was terminated by dilution with cold buffer and repeated centrifugation at  $40,000 \times g$  for 10 minutes. 1,4-DHP were first dissolved in absolute ethanol to give  $10 \text{ mmol.l}^{-1}$  and then diluted, to give the desired concentrations.

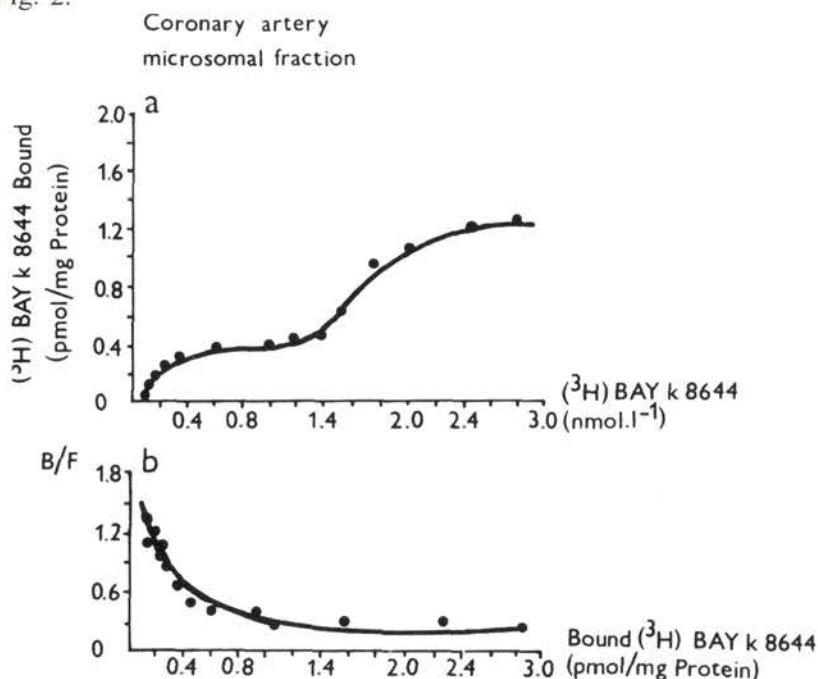
### *Drugs and Chemicals*

(5-methyl- $^3\text{H}$ )BAY k 8644) N. E. N. Research Product, DuPont; Nitrendipine (Bayer AG); Nitrendipine (Inst. Drug Res., Modra); Pentobarbital (Spofa), N-ethylmaleimide (Fluka); Dipyridamol (Boehringer); 2-deoxyadenosine (Sigma). All other chemicals were purchased in the highest grade of purity commercially available. All manipulations and procedures with 1,4-dihydropyridines were performed under sodium light.



## Results and Discussion

The dihydropyridine calcium channel agonist ( $^3\text{H}$ )BAY k 8644 binds rapidly to microsomal fraction of the coronary artery reaching a steady state within 40 minutes. The calculated association rate measured in kinetic experiments with  $0.91 \text{ nmol.l}^{-1}$  ( $^3\text{H}$ )BAY k 8644 was  $0.48 \text{ min}^{-1}$ . The specific binding of ( $^3\text{H}$ )BAY k 8644 to the microsomal fraction, measured in saturation experiments ranged between  $0.05$  and  $3.0 \text{ nmol.l}^{-1}$ , was saturable with two equilibria, one within a concentration range between  $0.05$  and  $1.0 \text{ nmol.l}^{-1}$  BAY k 8644, and another between  $1.2$  and  $3.0 \text{ nmol.l}^{-1}$ . Scatchard analysis of binding in the lower range of ( $^3\text{H}$ )BAY k 8644 concentrations suggested saturability of specific binding and one population of high affinity binding sites ( $K_D = 0.60 \pm 0.02 \text{ nmol.l}^{-1}$  and  $B_{\text{max}} = 0.76 \text{ pmol.mg}^{-1}$  protein). The analysis in the other concentration range ( $1.2 - 3.0 \text{ nmol.l}^{-1}$ ) revealed a population of low affinity binding sites ( $K_D = 9.09 \pm 0.1 \text{ nmol.l}^{-1}$  and  $B_{\text{max}} = 3.15 \text{ pmol.mg}^{-1}$  of protein) Table 1 and Fig. 2.



**Fig. 2.** a: Specific binding of ( $^3\text{H}$ )BAY k 8644 as a function of increasing concentrations of ( $^3\text{H}$ )BAY k 8644. Coronary artery microsomal membranes were incubated at  $25^\circ\text{C}$  with various concentrations of ( $^3\text{H}$ )BAY k 8644 (from  $0.05$  to  $3.0 \text{ nmol.l}^{-1}$ ). Nonspecific binding was measured in the presence of  $1.0 \text{ }\mu\text{mol.l}^{-1}$  BAY k 8644 or  $10 \text{ }\mu\text{mol.l}^{-1}$  of nitrendipine. The data shown are from 6 experiments, each performed in triplicate.

<sup>b</sup>Scatchard analysis of the specific binding.

**Table 1.** High and low affinity specific ( $^3\text{H}$ )BAY k 8644 binding to 1,4-dihydropyridine receptors associated with calcium channels in coronary artery microsomal membranes, and characteristics of low affinity binding sites

<i>a</i> Affinity State	$K_D(\text{nmol.l}^{-1})$	$B_{\text{max}}(\text{pmol.mg}^{-1} \text{ protein})$
High	0.60 $\pm 0.02$	0.76 $\pm 0.02$
Low	9.09 $\pm 0.09$	3.15 $\pm 0.18$
<i>b</i> Low Affinity Agonist Binding		
Procedure	$K_D(\text{nmol.l}^{-1})$	$B_{\text{max}}(\text{pmol.l}^{-1} \text{ protein})$
Concentration ( $\mu\text{mol.l}^{-1}$ )		
Lyophilization	10.11 $\pm 1.08$	3.09 $\pm 0.21$
N-ethylmaleimide (100)	9.57 $\pm 1.02$	0.85 <sup>s</sup> $\pm 0.16$
Dipyridamole (10)	9.18 $\pm 0.70$	1.63 <sup>s</sup> $\pm 0.15$
2-Deoxyadenosine (10)	8.07 $\pm 0.6$	1.03 <sup>s</sup> $\pm 0.14$

Plasma membranes were incubated with 100  $\mu\text{mol.l}^{-1}$  N-ethylmaleimide, or 10  $\mu\text{mol.l}^{-1}$  dipyridamole or 2-deoxyadenosine for 30 min. at 4°C. The reaction was terminated by dilution with cold buffer. The membranes were repeatedly washed and then incubated with 0.05–3.0  $\text{nmol.l}^{-1}$  of ( $^3\text{H}$ )BAY k 8644. Values are mean  $\pm$  SEM of six experiments in each group. Experiments in triplicate.

An analysis of the results using the affinity spectra method (Tobler and Engel 1983) also suggested the presence of two populations of binding sites for ( $^3\text{H}$ )BAY k 8644.

Preincubation of coronary microsomal proteins with-SH alkylating agent N-ethylmaleimide was followed by a decrease in the specific binding of ( $^3\text{H}$ )BAY k 8644. The decrease was due to a loss of the low affinity specific binding of ( $^3\text{H}$ )BAY k 8644, while both the nonspecific and the high affinity specific binding remained unchanged. The binding was reversible, the dissociation rate constant ( $k_{-1}$ ) of the drug-receptor complex in experiments with lyophilized microsomal membranes and excess of nitrendipine (10  $\text{nmol.l}^{-1}$ ) showed values of 0.042  $\text{min}^{-1}$  ( $n = 3$ ). Saturation analysis by Scatchard plots suggested that the decrease of low affinity binding was entirely attributable to an apparent decrease of the number of binding sites ( $B_{\text{max}}$ ) with both the affinity of binding sites and  $K_D$  remaining unchanged.

In order to explore the nature the ( $^3\text{H}$ )BAY k 8644 low affinity binding sites another series of experiments was designed to test the effects of nucleoside

transport inhibitors dipyridamole and 2-deoxyadenosine (Table 1b). Preincubation of the coronary artery microsomal fraction with 2-deoxyadenosine or dipyridamol significantly reduced ( $^3\text{H}$ )BAY k 8644 binding. Lyophilization and subsequent treatment of the microsomal fraction according to the experimental protocol had no effect on ( $^3\text{H}$ )BAY k 8644 binding.

The ( $^3\text{H}$ )BAY k 8644 high affinity binding sites in the coronary artery microsomal fraction described herein have properties similar to those of the 1,4-DHP receptor recognition sites in calcium channels in the vascular smooth muscle. The decrease of the number of low affinity binding sites following preincubation of membrane proteins with N-ethylmaleimide suggests that -SH groups may affect ( $^3\text{H}$ )BAY k 8644 binding to calcium channel agonist binding sites in the coronary artery microsomal fraction.

Although the high affinity 1,4-DHP binding site of the coronary artery investigated in the present study in saturation experiments with ( $^3\text{H}$ )BAY k 8644 seems to have similar characteristics as those described previously for bovine aorta (Sarmiento et al. 1984), canine aorta (Triggle et al. 1982) and the porcine coronary artery (Dompert and Traber 1984) the numbers of the high affinity binding sites seem to be 5 times higher in the canine coronary artery than  $B_{\text{max}}$  reported for the ( $^3\text{H}$ ) nitrendipine labeled binding sites in the porcine coronary artery. The proportions of high and low affinity binding sites in the coronary artery labeled by ( $^3\text{H}$ )BAY k 8644 (ligand with agonist properties) indicate that the difference in  $B_{\text{max}}$  values may be explained on the basis of labeling or stabilizing high affinity binding sites in the coronary smooth muscle. The second component of specific binding to the microsomal fraction of the coronary artery studied, exhibited a clear saturation at concentrations between 2 and 3 nmol.l<sup>-1</sup>. The Scatchard analysis of these data showed  $K_D$  values around 10 nmol.l<sup>-1</sup>, similar to the low affinity ( $^3\text{H}$ )BAY k 8644 binding sites described previously by Janis et al. (1984) in heart muscle, or in skeletal muscle (Glossmann et al. 1985).

Whether the high and low affinity ( $^3\text{H}$ )BAY k 8644 binding sites revealed in the coronary microsomal fraction in the present study concern different functional modifications of 1,4-DHP binding sites on the same calcium channel, or whether the low affinity binding sites derived from the neuronal membranes and membrane vesicles contaminating the microsomal fraction of the coronary smooth muscle, remains to be established.

A model of the slow calcium channel with two 1,4-dihydropyridine binding sites regulating the functions of the calcium channel has been proposed (Thomas et al. 1984). According to this model, it depends on the chemical structure and the concentration of the respective 1,4-DHP whether occupation of the second binding site (i. e. that with the low affinity, hidden by the high affinity 1,4-DHP binding site) will turn the channel to one with a low calcium permeability. Nevertheless, the reduction of the number of low affinity binding sites after



2-deoxyadenosine and dipyridamole treatment with the high affinity binding sites remaining unchaned, suggests that the low affinity ( $^3\text{H}$ )BAY k 8644 binding sites in the microsomal fraction of the coronary smooth muscle has properties that would indicate that the low affinity binding site may correspond to the „second 1,4 DHP site“ on the calcium channel.

It is concluded that microsomal membranes of the dog coronary artery smooth muscle contain two ( $^3\text{H}$ )BAY k 8644 binding sites, differing, a. o. in their participation. The existence of high, less numerous, and low affinity binding sites for ( $^3\text{H}$ )BAY k 8644 in the coronary artery would fit with the proposed model of 1,4-dihydropyridine binding sites being modulators of calcium channel conductance in vascular smooth muscle.

## References

- Domper W. U., Traber J. (1984): Binding sites for dihydropyridine calcium antagonists. In: Calcium Antagonists and Cardiovascular Disease (Ed. Opie L. H.), pp. 175—179, Raven Press, New York
- Dube G. P., Baik Y. H., Schwartz A. (1985): Effects of a novel calcium channel agonist dihydropyridine analogue BAY k 8644 on pig coronary artery. Biphasic mechanical response and paradoxical potentiation of contraction by diltiazem and nimodipine. *J. Cardiovasc. Pharmacol.* **7**, 377—389
- Fleckenstein A. (1977): Specific pharmacology of calcium in myocardium, cardiac pacemakers and vascular smooth muscle. *Annu. Rev. Pharmacol.* **17**, 149—166
- Glossman H., Ferry D. R., Goll A., Striessing J., Zernig G. (1985): Calcium channels and calcium channel drugs. Recent biochemical and biophysical findings. *Arzneim.-Forsch.* **35**, 1917—1935
- Grupp I. L., Dube G. P., Baik T. H. (1984): Effects of dihydropyridine  $\text{Ca}^{2+}$  agonist BAY k 8644 on porcine coronary artery, isolated rat hearts and canine Purkinje and trabecular action potentials. *Fed. Proc.* **4**, 43—50
- Hess P., Landsmann J. B., Tsien F. W. (1984): Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonist and antagonist. *Nature* **311**, 538—544
- Janis R. A., Raupe D., Sarmiento J. G., Triggle D. J. (1984): Specific binding of a calcium channel activator ( $^3\text{H}$ )BAY k 8644 to membranes from cardiac muscle and brain. *Biochem. Biophys. Res. Commun.* **121**, 317—323
- Kwam C. Y., Triggle C. R., Groover A. K., Lee R. M. K. W., Daniel E. E. (1983): An analytical approach to the preparation and characterization of subcellular membranes from canine mesenteric arteries. *Prep. Biochem.* **13**, 275—310
- Nyborg N. C. (1984): Effects of calcium antagonists on resistance vessels. A dihydropyridine derivative BAY k 8644 with agonistic properties on isolated rat coronary resistance vessels. *Progr. Appl. Microcirc.* **8**, 53—58
- Sarmiento G. J., James R. A., Jenkins D., Triggle D. J. (1984): Characteristics of ( $^3\text{H}$ ) nitrendipine binding to vascular smooth muscle membranes. In: Nitrendipine (Eds. Scriabine A., Vanor S., Beck J.), pp. 153—169, Baltimore, Milan
- Thomas G., Gross R., Schramm M. (1984): Calcium channel modulation. Ability to inhibit or promote calcium influx in the same dihydropyridine molecule. *J. Cardiovasc. Pharmacol.* **6**, 1170—1176

- Tobler H. J., Engel J. (1983): Affinity spectra: A novel way for the evaluation of equilibrium binding experiments. *Arch. Pharmacol.* **322**, 182—192
- Triggle C. R., Agrawal D. K., Bolger C. T., Daniel E. E., Kwam C. Y., Luchowski E. M., Triggle D. J. (1982): Calcium channel antagonist binding to isolated vascular smooth muscle membranes. *Can. J. Physiol. Pharmacol.* **60**, 1738—1741
- VanBreemen C., Farinas B. R., Gerba P., McNaughton E. D. (1972): Excitation-contraction coupling in rabbit aorta studied by the lanthanum method for measuring cellular calcium influx. *Circ. Res.* **30**, 44—45

Final version accepted July 14, 1987