Characteristics of [3H]Nimodipine Binding to Sarcolemmal Membranes from Rat Vas Deferens and Its Regulation by Guanine Nucleotide

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Abstract—The binding properties of a 1,4-dihydropyridine (DHP) calcium entry blocker, [3H]nimodipine, to a microsomal fraction from rat vas deferens was characterized. The specific binding was saturable, rapid and reversible. Scatchard analysis of the binding revealed a single binding site, and the dissociation constant and the maximum number of binding sites were 0.31±0.02 nM and 97.0±7.19 fmol/mg protein, respectively. Both the Kd value obtained from the kinetic study and the IC50 value from relaxation of the K+-depolarized organ were approximately 0.4 nM, indicating that the binding site is closely related to the functional Ca2+ channel. The specific [3H]nimodipine binding was displaced by DHP derivatives at low concentration and by verapamil at high concentration, but diltiazem had no effect on the binding. Calcium chelating agents decreased the [3H]nimodipine binding which was restored by adding Ca2+. 5'-Guanylylimidodiphosphate caused a rightward shift of the displacement curve for Bay K 8644 but not for nimodipine, suggesting the involvement of guanine nucleotide binding protein in the signal transduction between the DHP binding site and the Ca2+ channel.

It is generally considered that dihydropyridine (DHP) Ca2+ antagonists (or Ca2+ entry blockers) interact with Ca2+ channels, which are essential for the excitation-contraction coupling of cardiac and smooth muscles. Specific, reversible and high-affinity binding of DHPs has been identified in a variety of tissues including vascular and non-vascular smooth muscle (1-5). However, the correlation between the binding of radiolabelled DHPs to the Ca2+ channel and physiological block of Ca2+ channels is not always clear.

Recent electrophysiological studies have suggested the existence of more than one type of Ca2+ channels in neuronal tissues (6-8), cardiac (9, 10) and smooth muscle (11-13), which can be distinguished on the basis of differences in DHP-sensitivity. However, only a few reports showed more than one type of binding site for DHPs (14, 15).

Vasa deferentia smooth muscle have been used extensively for pharmacological studies as a good model for investigating sympathetic neurotransmission, and their adrenergic receptors have been characterized. However, the fundamental characteristics of DHP binding to this tissue have not been reported. A voltage clamp study from our laboratory using isolated single cells from rat vas deferens suggested the existence of only one type of Ca2+ channel whose sensitivity to DHP varied with membrane potential (16, 17). Therefore, it is interesting to correlate these results with the binding of DHP and relaxation by DHP.

Guanine nucleotide binding proteins (G proteins) are known to participate in various transmembrane signaling mechanisms such as adrenergic β- and α2-receptor-mediated effects (18, 19). Acetylcholine-induced opening of K+ channels in the heart is mimicked by intracellular application of G protein (20). Hescheler et al. (21) showed the involve-
ment of G protein in the depression of Ca\textsuperscript{2+} current by an enkephalin-analogue in neuroblastoma \times glioma hybrid cells. Therefore, it is possible that G protein plays a role in the regulation of Ca\textsuperscript{2+} channel function. However, the effect of G protein on the binding of DHP to Ca\textsuperscript{2+} channels is not known.

The purposes of the present study were (1) to elucidate basic properties of DHP binding to Ca\textsuperscript{2+} channels in sarcolemmal membranes from rat vas deferens and to compare them with the reported results for other smooth muscles and (2) to investigate the participation of G protein in DHP binding.

Materials and Methods

Preparation of microsomes for radioligand binding: Male Wistar rats weighing 200 to 400 g were killed by a blow on the head and subsequently exsanguinated. Vasa deferentia were removed immediately and placed in ice-cold 0.25 M sucrose buffer solution having the following composition: 250.0 mM sucrose, 1.0 mM MgCl\textsubscript{2}, and 5.0 mM tris-(hydroxymethyl)aminomethane (Tris), pH 7.4 at 4°C. After connective tissues and arteries were removed, vasa deferentia were minced with scissors and homogenized in sucrose buffer solution with a Polytron (Kinematica, Switzerland) for 5 sec, which was repeated 5 times with an interval of 30 sec. The homogenate was centrifuged at 2,500\times g for 10 min. The supernatant was centrifuged at 15,000\times g for 20 min, and the resultant supernatant was centrifuged again at 100,000\times g for 60 min. The microsomal pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) and stored at -80°C until use. Protein concentration was measured according to Lowry’s method (22).

Activity of a plasma membrane marker enzyme, 5’-nucleotidase, was measured to confirm that the microsomal fraction prepared as above was rich in sarcolemmal membrane. The microsomal protein suspension (0.1 ml) and 1 ml of 5 mM 5’-adenosine monophosphate were incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.25 ml of ice-chilled trichloroacetic-acid (25% w/v), and the reaction mixture was centrifuged at 3,000 rpm for 10 min. The content of inorganic phosphate in the supernatant was measured by the method of Fiske and Subbarow (23), with some modifications.

Specific activities of the crude homogenate and the final microsomes were 0.18 and 1.52 mmol Pi/mg protein/hour (mean of three experiments), respectively, which shows an 8-fold increase in purity.

[\textsuperscript{3}H] Nimodipine binding: The microsomal protein suspension was incubated with [\textsuperscript{3}H]-nimodipine in 0.2 ml of Tris-HCl buffer at 25°C for 20 min in a duplicate set of tubes. The incubation was performed in the dark to prevent decomposition of DHPs. The incubation was terminated by filtration through Whatman GF/F filters, which were then washed three times with 3 ml of ice-cold Tris-HCl buffer. The filters were dried, and radioactivity was measured in a toluene base scintillator with a liquid scintillation spectrometer (Aloka LSC-700). Non-specific binding was determined in the presence of 0.5 mM unlabelled nimodipine.

The specific binding increased linearly with increasing protein concentration over the range of 0.05 to 1.0 mg protein/ml. Therefore, binding assays were routinely carried out with 0.5 or 1.0 mg protein/ml.

We selected the incubation time of 20 min because, as shown in Fig. 2, the specific binding was nearly saturable, and the non-specific binding tended to increase with a longer period of incubation time.

Recording of mechanical response: Vasa deferentia were placed in physiological salt solution (PSS), and surrounding tissues were removed. The composition of the PSS was as follows: 118.0 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 1.2 mM NaH\textsubscript{2}PO\textsubscript{4}, 25.0 mM NaHCO\textsubscript{3} and 11.1 mM glucose. The vasa deferentia were mounted vertically in a 30 ml organ bath containing PSS. The PSS was gassed continuously with a mixture of 95%-O\textsubscript{2} and 5%-CO\textsubscript{2} and warmed to 37°C. The tissue was allowed to equilibrate for 60 min under a resting tension of 10 mN. Isometric contraction was measured with a force-displacement transducer (Nihon Kohden TB-612T) and recorded on an ink-writing oscillograph (Nihon Kohden WI621-G). High K\textsuperscript{+}-induced contracture of the vas deferens was elicited by displacing the normal PSS solution with high K\textsuperscript{+} solution (total K\textsuperscript{+} concentration:
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63.7 mM) made by substituting half of the NaCl in the PSS with KCl. Concentration-response curves for nimodipine were obtained by stepwise addition of concentrated solution. Prazosin (10\(^{-6}\) M) was added throughout the experiment to block the effect of norepinephrine possibly released from sympathetic nerve terminals.

**Sources of drugs:** \[^3\text{H}\]Nimodipine (specific activity: 138.5 Ci/mmol) was purchased from New England Nuclear. The sources of other drugs used were as follows: nimodipine, nifedipine, Bay K 8644 (Bayer); prazosin (Pfizer); d- and l-cis diltiazem (Tanabe); 5'-guanylylimidodiphosphate (GppNHp), verapamil (Sigma); ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis(2-aminoethylether)-N,N,N',N"-tetraacetic acid (EGTA), (Wako).

**Results**

**Specific binding of \[^3\text{H}\]nimodipine:** Figure 1 depicts the specific binding of \[^3\text{H}\]nimodipine to the microsomes. The binding was apparently monophasic and saturable, but it did not reach steady-state at 5 nM. When the concentration of the radioligand was over 5 nM, non-specific binding increased abruptly, and apparent specific binding was decreased. We increased unlabelled nimodipine concentration up to 5 mM, but the result was the same. Since it was difficult to determine the specific binding accurately, we did not analyze the specific binding at concentrations higher than 5 nM. Scatchard analysis of specifically bound \[^3\text{H}\]nimodipine yielded a straight line (Fig. 1 inset), indicating a single binding site. The dissociation constant (K\(_d\)) and the maximum number of binding sites (B\(_{\text{max}}\)) were 0.31±0.02 nM and 97.0±7.19 fmol/mg protein (mean±S.E.M., n=4), respectively. The Hill plot of the saturation data was linear, and the Hill coefficient (n\(_H\)) was 0.98±0.01.

**Kinetic study of the binding:** The rate constants for association (k\(_1\)) and dissociation (k\(_2\)) of \[^3\text{H}\]nimodipine binding were determined. The pseudo-first order rate constant (k\(_{\text{obs}}\)) for the association reaction was 0.176 min\(^{-1}\) (Fig. 2B). The dissociation rate constant (k\(_2\)) of 0.075 min\(^{-1}\) was determined from the slope of a first order rate plot (Fig. 2D). The association rate constant (k\(_1\)) was calculated from the following equation:

\[
k_1 = (k_{\text{obs}}-k_2)/([\text{\[^3\text{H}\]nimodipine}]) = 0.202 \text{min}^{-1} \cdot \text{nM}
\]

where ([\[^3\text{H}\]nimodipine]) refers to the concentration of \[^3\text{H}\]nimodipine (0.5 nM).

The ratio of k\(_2\)/k\(_1\), as an estimate of the equilibrium dissociation constant (K\(_d\)), was 0.37 nM, which was not significantly different from the one obtained from the saturation binding study.

**Mechanical response:** The pharmacological potency of nimodipine that elicited relaxation to K\(^+\) depolarized rat vas deferens was investigated. When normal PSS was replaced by high-K PSS, the vasa contracted rapidly and then relaxed to a new steady-state level. Nimodipine was added after the vasa reached the steady-state tonic phase. The muscle was relaxed in a concentration-dependent manner (Fig. 3). The IC\(_{50}\) value was 0.44±0.03 nM (n=5), which is in good agreement with the K\(_d\) value obtained from the binding study.

**Displacement of \[^3\text{H}\]nimodipine binding:** Several drugs classified as Ca\(^{2+}\) channel antagonists inhibited the specific \[^3\text{H}\]-nimodipine binding, and the most potent inhibitors were DHPs. Nimodipine and nifedipine inhibited the binding in a similar
Fig. 2. Kinetic analysis of $[3^H]$nimodipine binding in rat vas deferens. A, B: Association of the specific $[3^H]$nimodipine binding. The pseudo-first order rate constant of the association ($k_{obs}$) is equal to the slope of the regression line determined by the plot of $\ln(B_{eq}/(B_{eq}-B))$ against the incubation time, where $B$ is binding and $B_{eq}$ is the binding at equilibrium. The $k_{obs}$ value was 0.176 min$^{-1}$ in this example. C, D: Dissociation of the specific $[3^H]$nimodipine binding. Membranes were equilibrated with 0.5 nM $[3^H]$nimodipine for 30 min at 25°C, and then the dissociation was initiated by the addition of an excess amount of nimodipine (0.5 mM). The $k_2$ value calculated from the slope of the line in D was 0.075 min$^{-1}$.

Fig. 3. Concentration-response curve for the relaxation of $K^+$-depolarized muscle induced by nimodipine. Isolated vas deferens was depolarized by 63.7 mM $K^+$ PSS in the presence of $10^{-6}$ M prazosin, and nimodipine was added in the tonic phase of contraction in a cumulative manner. Each point and vertical bar represent the mean±S.E.M. from 5 experiments.

Fig. 4. Inhibition of the $[3^H]$nimodipine binding by various DHP derivatives. Various concentrations of nimodipine (filled circles), nifedipine (filled squares), and Bay K 8644 (filled triangles) were incubated with the microsomal membranes of rat vas deferens in the presence of 0.3 nM $[3^H]$nimodipine for 20 min at 25°C. The specific binding was determined as that displaced by $10^{-5}$ M nimodipine. Number of preparations was 3 to 8.
concentration range (10^{-10} to 10^{-7} M, Fig. 4). The IC50 values of nimodipine and nifedipine were 3.01±0.75 (n=7) and 3.25±0.96 nM (n=3), respectively. Bay K 8644, a DHP Ca^{2+} entry facilitator, inhibited the specific binding, but the displacement was slightly less potent than those by nimodipine and nifedipine (IC50: 35.4±5.84 nM, n=8). These results indicate that the specific binding site of [3H]-nimodipine was a common characteristic of the DHPs. However, the pseudo Hill coefficients of nimodipine, nifedipine and Bay K 8644 were 0.77±0.07, 0.70±0.13 and 0.74±0.04, respectively, which were all significantly smaller than unity.

Several investigators showed that verapamil depressed the DHP binding, whereas diltiazem increased it in various tissues (24–27). Therefore, we studied the effects of verapamil, d- and l-cis diltiazem on the [3H]nimodipine binding. Verapamil inhibited the specific binding at concentrations higher than 10^{-8} M (Fig. 5), and the IC50 value was 349.7 nM (n=4). In contrast, d- and l-cis diltiazem scarcely affected the binding even at 100 μM.

The effects of Cd^{2+} and Ca^{2+} chelators: The effects of Cd^{2+} and Ca^{2+} chelating agents, EDTA and EGTA, on specific [3H]-nimodipine binding were examined. EDTA decreased the binding concentration-dependently, and this decrease was attenuated in the presence of 1 mM Ca^{2+} (Fig. 6). Similar results were obtained in the study using EGTA instead of EDTA, but 10 mM Ca^{2+} was necessary to restore the binding. Cadmium (1 mM) significantly decreased the binding which was not restored by the addition of 10

Fig. 5. Inhibition of the [3H]nimodipine binding by verapamil (filled squares), and d- and l-cis diltiazem (unfilled squares). The experimental conditions were similar to those described in Fig. 4. Since there was no significant difference in the results between d- and l-cis diltiazem, the data were combined. The data are the means of 4 experiments.

Fig. 6. Effects of 10^{-5} and 10^{-3} M EDTA, EGTA and Ca^{2+} on the [3H]nimodipine binding (0.3 nM) in the absence (open column) or the presence (stippled column) of Ca^{2+} ions. The specific binding was determined as that displaced by 10^{-5} M nimodipine. The data are from 3 to 6 experiments.

Fig. 7. Effects of GppNHp on the displacement curves of nimodipine (circles) and Bay K 8644 (triangles) for [3H]nimodipine binding. Competition for [3H]nimodipine binding was established in the absence (filled symbols) or the presence (unfilled symbols) of GppNHp (10 μM). Specific binding was determined as that displaced by 10^{-5} M nimodipine. The displacement curves in the absence of GppNHp were the same as in Fig. 4, which were redrawn for comparison. Number of preparations in the presence of GppNHp was 4.
mM Ca\(^{2+}\). Magnesium or Ca\(^{2+}\) at the concentration of 1 mM did not affect the binding significantly.

The effect of GppNHp on the \([\text{H}]\)nimodipine binding: The effect of GppNHp (10 \(\mu\)M) on the displacement curves of nimodipine and Bay K 8644 were investigated. Displacement curve of nimodipine was not affected by GppNHp, but that of Bay K 8644 was shifted to the right significantly and the IC\(_{50}\) values were increased to about 15-fold (Fig. 7). The Hill coefficients of the curves did not change significantly.

Discussion

The present study showed that the binding of \([\text{H}]\)nimodipine to the microsomal preparation from rat vas deferens was saturable, rapid and reversible; and the binding site was common to the DHP derivatives. The dissociation constant (\(K_d\)) obtained from equilibrium binding and kinetic studies was in the range of 0.3 to 0.4 nM, which is about the same as the reported \(K_d\) values in other smooth muscle membranes: 0.26 nM for rat ileum (25), 0.30 nM for guinea pig ileum (28), 0.16 nM for bovine aorta (29), 0.36 nM for rat tail artery (30), 0.25 nM for canine mesenteric artery (31), 0.31 nM for canine aorta (31) and 0.10 nM for rat mesenteric artery (31). Slightly higher \(K_d\) values of 1.6 nM for pig coronary artery (24) and 1.7 nM for guinea pig ileal longitudinal muscle (30) have also been reported. In the case of cardiac muscle, the equilibrium dissociation constant does not match the concentration of DHP needed for a physiological effect on the membrane. Therefore, several investigators suggested the presence of a low-affinity as well as a high-affinity binding site for DHP to explain the discrepancy (14, 15). Rogart et al. (15) showed a low-affinity (\(K_d\): 235 nM) binding site in bovine aorta which occupied 99% of the total nitrendipine binding. Although we could not analyze the specific binding when the concentration of the radioligand was higher than 5 nM, Scatchard analysis up to 5 nM indicated a single binding site for DHP. Therefore, the low-affinity binding site in rat vas deferens, if it exists, will be very small or its \(K_d\) value will be much higher than \(10^{-8}\) M. There is a very good correlation between the affinity of DHPs for the nimodipine binding sites and the tonic portion of \(K^+\) induced muscle contraction. A voltage-clamp study on single isolated cells from rat vas deferens indicated that the IC\(_{50}\) value of nicardipine determined from the block of the slowly decaying component of Ca\(^{2+}\) channel current was 16 nM (17). However, this value may be overestimated or the effects were underestimated, because the effect of nicardipine was determined for a short period of time to avoid “run down” of the current. Effects of DHPs on Ca channel current are known to be voltage-dependent (32–35) and increase at more positive potentials. Both the microsomal fraction and high K\(^+\) contracted muscle have no or very small membrane potential, and the affinity of DHP will be increased under these conditions. Based on these results from three different kinds of studies, we conclude that DHP binds to the high-affinity site to produce physiological effects in rat vas deferens.

A paradoxical increase in DHP binding by d-cis-diltiazem, but not by l-cis-diltiazem, has been reported using microsomes from pig coronary artery (24), guinea pig ileum (26), human myometrium (36) and various other tissues (1). However, in the present study, verapamil displaced the \([\text{H}]\)nimodipine binding, whereas diltiazem showed no effect. Therefore, allosteric potentiation of DHP binding by diltiazem may not be common to all of the tissues. There may be species or organ specific differences in the structure around the DHP binding site.

Calcium chelating agents, EDTA and EGTA, decreased the \([\text{H}]\)nimodipine binding, which was partially prevented by the addition of 1 mM or 10 mM Ca\(^{2+}\). The effect of Ca\(^{2+}\) on DHP binding is controversial. The DHP binding was dependent on the presence of Ca\(^{2+}\) ions in membranes from guinea pig ileum (26, 37) and rat brain (38). However, Fosset et al. (39) showed that a low concentration of Ca\(^{2+}\) (up to 1 mM) did not affect the binding, but a higher concentration decreased it in rabbit skeletal muscle. Galizzi et al. (40) concluded that the inhibitory effect of EDTA on DHP binding in skeletal muscle is not due to the removal of Ca\(^{2+}\) because other Ca\(^{2+}\) chelators such as
hydroxy-EDTA or EGTA (up to 100 μM) did not affect the binding. In our present study, 10^{-3} M EGTA inhibited the binding, which was partially restored in the presence of 10 mM Ca^{2+}. Therefore, it is possible that the inhibition by EDTA or EGTA is due to the Ca^{2+} chelating action of the chelators rather than their direct effect on the DHP binding. Relatively low concentration of Ca^{2+} ions may be necessary for DHP binding. Cadmium strongly depressed the binding, which was not affected by the addition of ten-times higher concentration of Ca^{2+}. The results may indicate either that Cd^{2+} and Ca^{2+} bind different sites or that Cd^{2+} has greater affinity to the effective site than Ca^{2+}.

Nimodipine and nifedipine inhibited the binding at the same low concentration (10^{-10} to 10^{-7} M), but the potency of Bay K 8644 was weaker than those of these DHPs. Radiolabelled Bay K 8644 binds to the tissues at relatively higher concentration in cardiac muscle (K_d is 2.5 to 35 nM) (41). These results support the notion that Bay K 8644 is a partial agonist.

The pseudo Hill coefficients obtained from the ligand competition study were less than unity, which may indicate that the binding of nimodipine is not simple one to one binding. Plausible explanations may be 1) existence of more than one type of binding site, 2) cooperativity among the binding sites, and 3) involvement of a G protein. However, as mentioned above, the analysis of the saturation of [3H]nimodipine suggested one to one binding to a single high-affinity site without cooperativities.

Guanine nucleotides are known to diminish the affinity of several types of receptors for their corresponding agonist, which are coupled to the adenylate cyclase (18, 19). The effects of guanine nucleotides on this type of receptor are the rightward shift of the displacement curves for the agonists and the increase in the pseudo Hill coefficient to a value close to 1. The dissociation constant of the low affinity binding site is about 100 times as large as that of the high affinity site. Receptors not coupled to adenylate cyclase, such as α1-adrenoceptors, are also regulated by a guanine nucleotide binding protein (G protein) (42-44). In these receptor-effector systems, guanine nucleotides shift the displacement curve to the right, but the shift is relatively small, and the pseudo Hill coefficient does not reach 1. The change of K_d value in the absence or the presence of guanine nucleotides is less than 10 times.

In the present study, the displacement curves for Bay K 8644, but not those for nimodipine, were shifted rightward by the addition of 10 μM GppNHp. Supposing that Bay K 8644 is an agonist for the DHP binding site and nimodipine is an antagonist, it is reasonable that the DHP binding site is equivalent to the general type of receptor whose binding site is regulated by a G protein. However, in the present study, the increase in IC50 value was about 15-fold, and the pseudo Hill coefficient was not affected by GppNHp. Therefore, the regulation may be similar to that observed in the α1-adrenoceptor-effector system. Although the present finding suggests the involvement of a G protein between the DHP binding site and Ca^{2+} channel, it was not the reason why the pseudo Hill coefficients of the displacement curves deviated from unity. Further studies are necessary to elucidate the precise mechanism for how the physiological function of the Ca^{2+} channel is regulated by a G protein.

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