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Dihydropyridine Binding and Calcium Channel Function in Clonal Rat Adrenal Medullary Tumor Cells

DIANA L. KUNZE,[1] SUSAN L. HAMILTON,[1] MARY J. HAWKES,[1] and ARTHUR M. BROWN[1]

Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

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SUMMARY

We correlated the binding of the dihydropyridines, nitrendipine and PN200-110, with their pharmacological actions on voltage-dependent membrane calcium channels. Binding was studied in clonal rat adrenal medullary cells (PC12) and in plasma membranes prepared from them. Calcium currents were studied using whole cell and single channel patch clamp methods. For both [^{3}H]-(±)-nitrendipine and [^{3}H]-(+)-PN200-110, high affinity binding sites with dissociation constants of 0.6 and 0.04 nM, respectively, were identified both in membrane fragments and in intact cells. In crude membrane preparations a low affinity nitrendipine-binding site was also found. The dissociation constant for binding at this site was affected by ionic strength and the presence of divalent cations. In 500 mM KCl, 0.1 mM $CaCl_2$, 50 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.4), the K_D is about 70 nM. The number of high affinity binding sites for dihydropyridines was between 30 and 100 fmol/mg of protein while the number of low affinity sites was between 30 and 70 pmol/mg of protein. In whole cells the measured number of high affinity sites was between 2000 and 4000/cell and, by extrapolation from the membrane preparation, the low affinity sites correspond to several million sites per cell. The electrophysiological effects of both of the dihydropyridines on Ca^{2+} currents were voltage dependent. When nitrendipine was applied, a small increase in calcium current occurred and this was followed by a decrease. The inhibitory effect was more pronounced at depolarized membrane holding potentials and was relieved by hyperpolarizing the membrane, whereas the stimulatory effect was pronounced at negative membrane holding potentials. In 10 nM nitrendipine these effects were also observed in single channels; they were not due to changes in channel conductance or dwell times in the open state but, rather, were due to changes in the probability of opening. The half-maximal inhibitory concentration (IC_{50}) for nitrendipine was 67 nM and the IC_{50} for the effect of (+)-PN200-110 was 9 nM using protocols which favored the depolarized state of the channel. No excitatory effect was seen. The IC_{50} from electrophysiological estimates is higher than the K_D for the high affinity binding site for both compounds. Using a simple model of voltage-dependent binding, we could not account for the difference. The number of functional channels calculated from the relation between whole cell and single channel calcium currents and the probability of opening was in good agreement with the number of high affinity sites calculated from the binding studies. In contrast, about 1000 low affinity binding sites are present per functional Ca channel; therefore, the low affinity sites are either unrelated to the Ca channel, include non-calcium channel molecules, or represent a pool of nonfunctional channels.

Voltage-dependent calcium channels regulate the entry of Ca^{2+} into the cell cytoplasm, thereby controlling a variety of biological processes including neurotransmitter release, muscle contraction, and hormone secretion. Dihydropyridines can either block or stimulate calcium entry through these channels in cardiac and smooth muscle tissue. They are now used clinically to treat a broad array of disorders related to calcium channel function and are being used biochemically in the isolation of the calcium channel and in the identification of the channel in solubilized membranes (1–4). In comparison to cardiac or smooth muscle, neuronal tissue appears to be relatively insensitive to the dihydropyridines. Calcium currents of *Helix* neurons are only 30% blocked by nifedipine at a concentration of 0.8 μM (5). ^{45}Ca uptake into KCl depolarized synaptosomes is not blocked at concentrations of nitrendipine up to 30 μM (6) or 100 μM (7) when external calcium is within normal values of 1–2 mM. Potassium-induced [^{3}H]dopamine release from cultured midbrain neurons, a calcium influx-dependent process, is similarly insensitive to nitrendipine (8). There are other studies, however, which suggest that some neuronal and neuroendocrine channels are more sensitive to dihydropyridines. Toll (9) has reported that potassium-stimulated ^{45}Ca uptake in PC12 cells is inhibited by nitrendipine with an IC_{50} of 5.5 nM. Scatchard analysis of [^{3}H]-(±)-nitrendipine binding

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[1] Present address: Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.

ABBREVIATIONS: MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

to membranes derived from these cells revealed a class of binding sites with a K_D of 1.1 nM and a B_{max} of 27.5 fmol/mg. Takahashi and Ogura (10) reported an IC_{50} value of 20 nM for nicardipine and a K_D for [^{3}H]-(±)-nitrendipine binding of 0.15 nM in similar experiments. Enyeart and Hinkle (11) reported that dihydropyridines completely inhibited a calcium-induced secretion of prolactin from a clonal line of pituitary neurons at 10 nM. An added complication is that nitrendipine may bind to more than one site. [^{3}H]-(±)-Nitrendipine binding to spinal cord neurons revealed two classes of sites: one with K_D of 0.3–1.1 nM and a second with a K_D of 22–50 nM (12). Toll (9) has suggested the possibility of a lower affinity site in PC12 cells although it was not characterized. Low affinity sites have also been found in cardiac and vascular smooth muscle (13–15).

The present study was undertaken to compare the binding of dihydropyridines with their electrophysiological effects on the same neuroendocrine cell membranes. We used PC12 cells because both nitrendipine binding (9) and single channel and whole cell calcium currents have been measured in these cells (16). We found that binding was complicated by the presence of large numbers of low affinity sites. There was, however, good agreement between the number of high affinity sites and the number of functional Ca channels. The electrophysiological effects were also complicated and could be stimulatory or inhibitory depending on membrane potential. At the molecular level the effects were on gating; changes in channel conductance did not occur.

Materials and Methods

Biochemical Studies

Chemicals. [^{3}H]-(±)-Nitrendipine (72.5 Ci/mmol) was obtained from New England Nuclear. [^{3}H]-(+)-PN200-110 (85 Ci/mmol) was purchased from Amersham. (±)-Nitrendipine was generously supplied by Miles Laboratories.

Cell cultures. The clonal rat pheochromocytoma line, PC12, was grown on plastic culture dishes at 37° in Dulbecco's modified Eagle's medium. The cells were subcultured and harvested at confluency. For use in whole cell binding assays, the medium was aspirated off, and the PC12 cells were removed from the plates, washed two times, and resuspended in binding buffer (pH 7.4). Aliquots were taken for cell counts and protein analysis.

Membrane isolation. PC12 cells from 50 confluent cell plates were washed two times with 150 mM NaCl, 50 mM sodium phosphate buffer (pH 7.4), to remove the medium. The cells were incubated in 5 mM Tris·HCl (pH 8.0) for 30 min on ice and then hand-homogenized. The lysed cells were centrifuged at 3,000 × g for 10 min to remove mitochondria and nuclei. The supernatant was centrifuged at 50,000 × g for 30 min. The white pellet was resuspended in 8.0 ml of a modified Krebs-Ringer buffer (buffer I) or in 50 mM MOPS (pH 7.4), and stored in liquid nitrogen. Buffer I consisted of 60 mM sucrose, 10 mM glucose, 130 mM NaCl, 4.8 mM KCl, and 25 mM MOPS (pH 7.4). From 50 confluent plates, an average of 32 mg of protein was obtained. Attempts to further purify the PC12 membranes by layering over sucrose gradients did not significantly improve the [^{3}H]-(±)-nitrendipine binding.

Radioligand binding. PC12 membranes (0.05–0.20 mg) were incubated in 2.0 ml of 0.1 mM $CaCl_2$, 50 mM MOPS (pH 7.4) at 25° for 2 hr with varying concentrations of [^{3}H]-(±)-nitrendipine (0.03–0.75 nM) or [^{3}H]-(+)-PN200-110 (0.005–0.36 nM). For measurement of nonspecific binding, unlabeled nitrendipine was present at a final concentration of 1 μM. Binding was terminated by rapid filtration on Whatman GF/B or GF/F glass fiber filters. The filters were washed five times with 5 ml of ice-cold water and counted in 10 ml of a Beckman HP/b scintillant. In assays where higher [^{3}H]-(±)-nitrendipine concentrations were used (10–500 nM), the specific activity of [^{3}H]-(±)-nitrendipine was decreased by a dilution (1:100) with unlabeled nitrendipine to 0.725 Ci/mmol. Nonspecific binding was determined in the presence of 10 μM unlabeled nitrendipine. Binding of [^{3}H]-(±)-nitrendipine to the filters alone was shown to be independent of unlabeled nitrendipine in both concentration ranges. All assays were done in duplicate or triplicate (as indicated) under red lights due to the light sensitivity of the dihydropyridines.

For PC12 whole cell binding, higher protein concentrations (0.2–0.4 mg/ml) were used. [^{3}H]-(±)-Nitrendipine (0.05–0.75 nM) or [^{3}H]-(+)-PN200-110 (0.002–0.5 nM) was incubated in triplicate with intact, unattached PC12 whole cells in 2.0 ml of 150 mM KCl, 0.1 mM $CaCl_2$, 5 mM NaCl, 50 mM sodium phosphate buffer (pH 7.4) in the presence and absence of 1 μM unlabeled nitrendipine for 60 min at 25°. Binding was terminated by rapid filtration on Whatman GF/A or GF/F glass filters with five washes of 5 ml of ice-cold buffer.

For determination of the association rate constants for dihydropyridine binding, PC12 membranes (0.2 mg/ml) were added to 0.1 mM $CaCl_2$, 50 mM MOPS (pH 7.4) containing [^{3}H]-(+)-PN200-110 at final concentrations of 0.17, 0.20, 0.25, and 0.45 nM. One-ml aliquots were filtered as a function of time after the addition of membranes. Nonspecific binding was determined in the presence of 1 μM unlabeled nitrendipine. Equilibrium values were obtained after 2-hr incubations at room temperature (25°). Upon reaching equilibrium, dissociation rate constants were determined by filtering 1-ml aliquots as a function of time after the addition of unlabeled dihydropyridine to 1 μM. All filtrations were through GF/F filters and were washed five times with 5 ml of ice-cold water.

Measurement of ATPase Activity

Mg^{2+}ATPase activity was determined by a modification of the procedure of Besch *et al.* (17). PC12 membranes (50 μg) were incubated in 0.5 ml of 3 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl, 50 mM Tris·HCl (pH 7.4), 3 mM Na_2ATP for 15 min at 37°. To determine Na^+/K^+ ATPase activity, 1 mM ouabain was included in the incubation mixture and the activity was defined as the difference in ATPase activity in the presence and absence of ouabain. Reactions were terminated by the addition of 1 ml of ice-cold 10% trichloroacetic acid. Following removal of denatured protein by centrifugation, duplicate aliquots of the supernatant were assayed for inorganic phosphate as described by Fiske and Subbarow (18).

Data analysis. The binding data were fit to a single-site model based upon the one-to-one occupancy of the Langmuir adsorption isotherm. Two-site models with independent sites were also studied. The program used a nonlinear least squares Marquardt method (19) to estimate the two free parameters, B_{max} and K_D. Parameter uncertainties were provided by χ^2.

Electrophysiological Studies

The patch clamp technique for intracellular recording was used to obtain whole cell calcium currents (20). Pipettes used for intracellular recording (1–5) MΩ) were filled with the following solution, in mM: 140 $CsCl_2$, 2 $MgCl_2$, 10 HEPES, 1 EGTA. pH was adjusted to 7.2 with KOH. In some studies EGTA was increased in 11 mM and 1 mM $CaCl_2$ was added. The extracellular solution contained, in mM: 10 $CaCl_2$, 20 tetraethylammonium chloride, 5 4-aminopyridine, 5 KCl, 10 HEPES, 10 glucose, and 85 Tris·HCl. pH was adjusted to 7.35 with KOH. In some experiments 40 or 20 mM $CaCl_2$ was used in the extracellular solution with an appropriate adjustment in Tris·HCl to maintain osmolarity. Nitrendipine and PN200-110 were first dissolved in ethanol or polyethylene glycol (PEG 400) to a concentration of 10 mM. Further dilution was made in the extracellular solution. Single calcium channels were recorded in the cell-attached configuration. For these studies, an extracellular solution containing 40 mM $CaCl_2$ was used in both the patch pipette and the bath.

Data collection and analysis. Whole cell and single channel cell currents were digitized on-line at 50 μsec/pt. The data were prefiltered at 5 kHz. The capacity transient was adjusted using a transient can-

cellation circuit. In the case of whole cell recordings any remaining transient was subtracted during analysis using scaled hyperpolarizing pulses. For single channel records (or samples) the capacitative artifact and the seal leak were corrected by subtraction of an averaged record of 10–15 samples without channel activity (failures). Single channel currents were further filtered to 800 or 1000 Hz during the analysis using a four-pole zero phase digital filter. Single channels were measured using the computer methods described by Lux and Brown (21) in which transitions and amplitudes were detected using a threshold discriminator set at 3–5 times the background noise level.

Results

[^{3}H]-(±)-Nitrendipine and [^{3}H]-(+)-PN200-110 binding to plasma membranes. Plasma membranes were isolated as described in Materials and Methods and showed about a 2- to 3-fold purification of ouabain-sensitive Na^+/K^+ ATPase activity relative to whole cells. At low concentrations of [^{3}H]-(±)-nitrendipine or [^{3}H]-(+)-PN200-110 and in 50 mM MOPS (pH 7.4), 0.1 mM $CaCl_2$, there is a single class of binding sites with an apparent dissociation constant of 0.56 ± 0.16 nM and a B_{max} of 91 ± 50 fmol/mg of protein (n = 13) for [^{3}H]-(±)-nitrendipine and a K_D of 0.029 ± 0.013 nM and a B_{max} of 41 ± 10 fmol/mg (n = 3) for [^{3}H]-(+)-PN200-110. Data from a single experiment are shown in Fig. 1. The nitrendipine experiments were performed over an 18-month period using cells maintained in continuous culture. During this time, there was a tendency toward a decrease in the number of binding sites. The PN200-100 experiments were performed over the latter 6 months of the period and reflect the lower value. In identical membranes the B_{max} values for [^{3}H]-(+)-PN200-110 and [^{3}H]-(±)-nitrendipine were identical (Fig. 1). The binding of [^{3}H]-(±)-nitrendipine and [^{3}H]-(+)-PN200-110 to membranes was linear up to 200 μg of protein, and all assays were carried out in the linear range. Nonspecific binding was again determined in the presence of 1.0 μM unlabeled nitrendipine. Nonspecific binding was also examined in the presence of 1 μM nimodipine and 1 μM nifedipine and was identical to that found with nitrendipine.

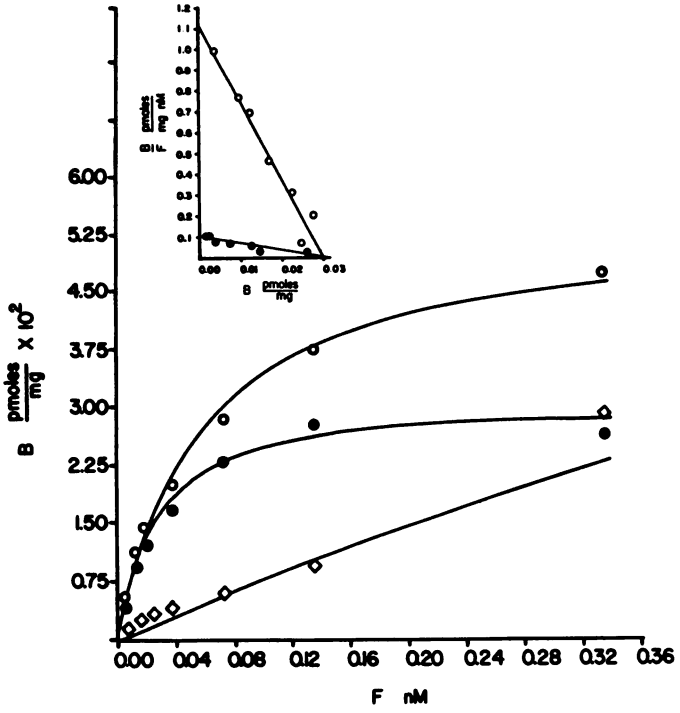

Fig. 1. [^{3}H]-(±)-PN200-110 binding to PC12 membranes at high affinity site. [^{3}H]-(±)-PN200-110 was incubated in duplicate in 2.0 ml of 50 mM MOPS (pH 7.4) with 90 μg of PC12 membranes in the presence and absence of 1 μM unlabeled nitrendipine. The curve of the specific binding (●) is a computer-derived fit of the data with a K_d = 0.026 nM and B_{max} = 30 fmol/mg for [^{3}H]-(+)-PN200-110. ○, total binding; ◇, nonspecific binding. *Inset*: Scatchard analysis of these data (0.004–0.5 nM) yields K_d = 0.026 nM and B_{max} = 30 fmol/mg for [^{3}H]-(±)-PN200-110 (○). Also shown is the Scatchard analysis of [^{3}H]-(±)-nitrendipine (0.03–2 nM) to these membranes (●) and yields a K_d of 0.3 nM and a B_{max} of 32 fmol/mg.

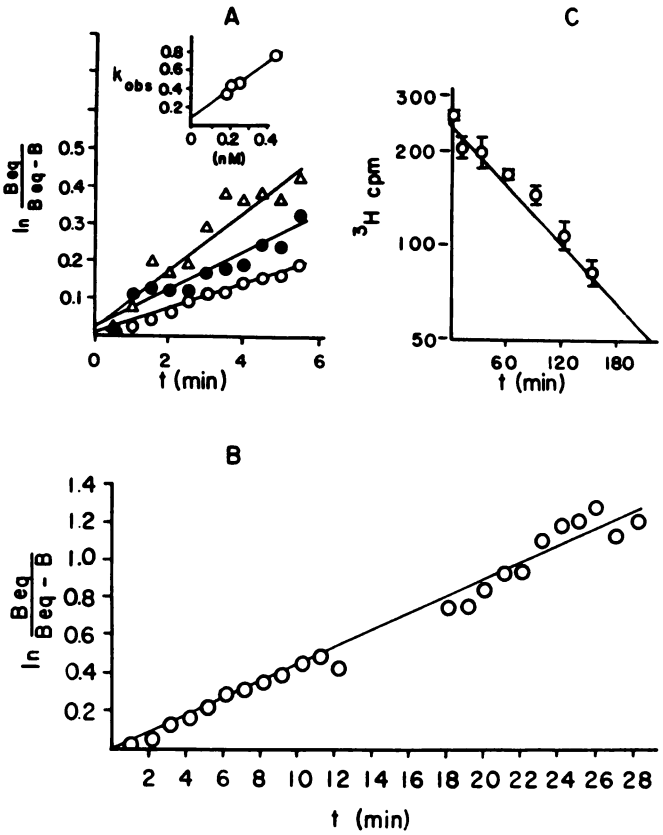

Fig. 2. Kinetics of [^{3}H]-(±)-PN200-110 binding. One-ml aliquots of PC12 membranes (0.2 mg of protein/ml) were filtered through Whatman GF/F filters as a function of time after addition of membranes to 0.17 (○), 0.25 (●), and 0.45 (△) nM [^{3}H]-(+)-PN200-110. Equilibrium binding was determined after 2 hr at room temperature. A. Binding as function of time after addition of membranes where B_{eq} = specific binding at equilibrium, B = specific binding at time t. *Inset*: k_{obs} versus concentration of radioligand. k_{obs} was determined as the slope of the plot shown in A. B. Binding as a function of time extended to about 30 min after addition of membranes to 0.02 nM [^{3}H]-(±)-PN200-110. C. Offrate. One-ml aliquots of PC12 membranes incubated with 0.2 nM [^{3}H]-(±)-PN200-110 for 2 hr at room temperature were filtered as a function of time after the addition of unlabeled nitrendipine to 1 μM.

Dissociation constants were also determined kinetically. The association rate constant (k_1) for [^{3}H]-(±)-nitrendipine binding was calculated to be 1.8×10^8 M^{-1} min^{-1}. The dissociation rate constant (k_{-1}) was found to be 0.06 ± 0.02 min^{-1} (n = 6). The kinetic data for [^{3}H]-(+)-PN200-110 are shown in Fig. 2. From a plot of k_{obs} as a function of [^{3}H]-(+)-PN200-110 concentration (Fig. 2A, *inset*), we calculate an association rate constant (k_1) of 1.56×10^8 M^{-1} min^{-1}. The y intercept of this plot which estimates the dissociation rate constant is about 1×10^{-2} min^{-1}. The value of the offrate constant is in reasonable agreement with the value determined experimentally (Fig. 2C) of 6.1×10^{-3} min^{-1}. This value is the average from two experiments, in

TABLE 1
Summary of equilibrium and kinetic data for intact cells and plasma membranes

Radioligand	Intact cells	
	K_d	B_{max}
	nM	fmol/mg
[^{3}H]-(±)-Nitrendipine	0.63 ± 0.31	55 ± 22 (n = 4)
[^{3}H]-(±)-PN200-110	0.044 ± 0.012	26 ± 4 (n = 3)

Radioligand	Plasma membranes				
	k_{-1}	k_1	Kinetic K_d	Eq K_d	B_{max}
	min^{-1}	M^{-1} min^{-1}	nM	nM	fmol/mg
Nitrendipine	0.060	1.8×10^8	0.33	0.56 ± 0.16	91 ± 50 (n = 13)
PN200-110	0.0061	1.56×10^8	0.039	0.029 ± 0.013	41 ± 10 (n = 3)

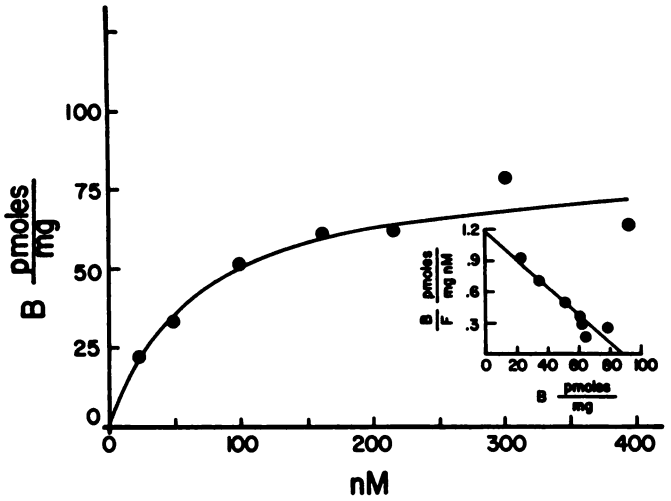

Fig. 3. Specific [^{3}H]-(±)-nitrendipine binding to PC12 membranes at low affinity site. PC12 membranes (50 μg) were incubated with [^{3}H]-(±)-nitrendipine, diluted 1:100 with unlabled nitrendipine, over a range of 25–400 nM in 1.0 ml of 0.1 mM $CaCl_2$, 500 mM KCl, 50 mM MOPS (pH 7.4) for 3 hr at room temperature. Nonspecific binding was defined as that binding not displaced by 10 μM unlabeled nitrendipine. The curve for specific [^{3}H]-(±)-nitrendipine binding is a computer-derived fit of the data using a one-site model with K_D = 68 nM and B_{max} = 84 pmol/mg. *Inset*: Scatchard analysis of the data yields K_D = 75 nM and B_{max} = 87 pmol/mg (r = 0.94).

each of which the amount bound at each time point was determined from triplicate incubations as shown in Fig. 2C. Biphasic association rates such as those reported by Weiland and Oswald (22) were not seen in these experiments even after longer incubation times (Fig. 2B). However, because of the high levels of nonspecific labeling, higher concentrations of [^{3}H]-(+)-PN200-110 such as those used by these workers were not examined. The use of the directly determined k_{-1} and k_1 (from the slope of k_{obs} versus ligand concentration) rate constants to calculate the K_D for [^{3}H]-(+)-PN200-110 gives a value of 0.039 nM, close to the value obtained by equilibrium binding. The K_D for [^{3}H]-(±)-nitrendipine from the kinetic data is 0.33 nM. The equilibrium and kinetic data are summarized in Table 1.

At high ionic strength and at nitrendipine concentrations well beyond the asymptotic maximum of the high affinity fit, a second class of nitrendipine binding sites is seen in isolated membranes (Fig. 3). The magnitude of binding to this site is ionic strength dependent but is maximal at 400 mM KCl. This site has not been detected in intact cells. In the presence of 0.1 mM $CaCl_2$, 500 mM KCl, 50 mM MOPS, the low affinity site has an apparent dissociation constant of 72 ± 10 nM and a B_{max} of 70 ± 30 pmol/mg (n = 3). It should, however, be recognized that these values are subject to error if the site is characterized by a rapid dissociation, since filtration would need to be completed within 0.1 sec to avoid significant loss of binding due to this dissociation (23). Preliminary kinetic analysis of binding to this site suggests, however, that the K_D is high due to a slow association rate rather than to a rapid dissociation rate. In addition, to minimize the dissociation the filters were washed with ice-cold water. The B_{max} value obtained corresponds to greater than 10^6 sites/cell. As the B_{max} for the low affinity site is considerably higher than the B_{max} for the high affinity site, the contribution of binding to the low affinity site is a significant part of the total binding under the assay conditions where assays for high affinity binding are performed at high ionic strength (Fig. 4). As can be seen in this figure, where binding obtained at low ionic strength (Fig. 2) is compared to that obtained at high ionic strength, high affinity binding cannot be determined in high ionic strength solutions.

Dihydropyridine binding to intact PC12 cells. The binding of low concentrations of [^{3}H]-(±)-nitrendipine to intact PC12 cells reached equilibrium after 1 hr incubation at room temperature. The membrane potential of cells measured after 1 hr in the incubation medium (150 mM NaCl, 50 mM Na_2HPO_4,

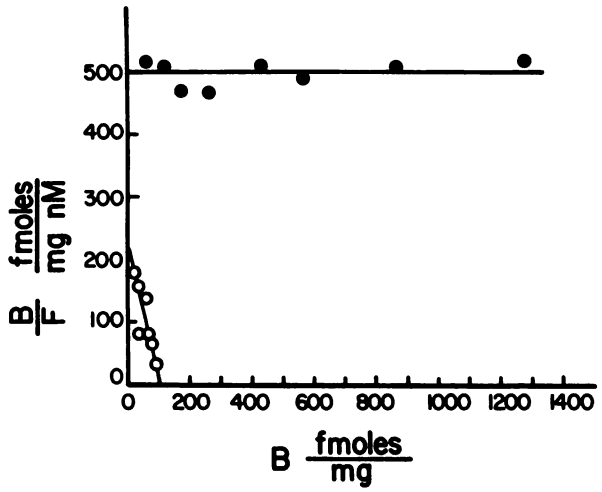

Fig. 4. Specific [^{3}H]-(±)-Nitrendipine binding to PC12 membranes at high affinity site in high and low ionic strength solutions. Binding was performed as described in Materials and Methods in high ionic strength solution, 0.1 mM $CaCl_2$, 500 mM KCl, 50 mM MOPS (pH 7.4) (●), and in low ionic strength, 0.1 mM $CaCl_2$, 50 mM MOPS (pH 7.4) (○). Nonspecific binding was that not displaced by 1 μM unlabeled nitrendipine. Scatchard analysis of the data obtained at low ionic strength yields K_d = 0.47 nM, B_{max} = 106 fmol/mg (r = 0.92).

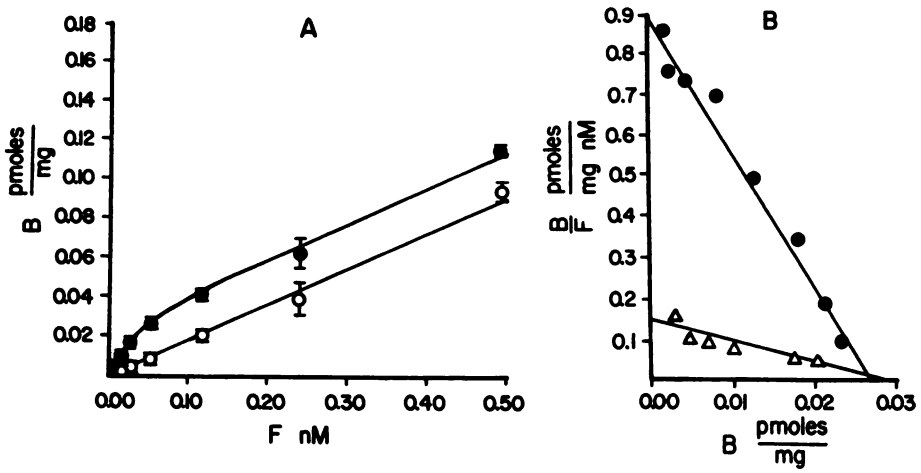

Fig. 5. Dihydropyridine binding to PC12 whole cells. A. [^{3}H]-(+)-PN200-110 (0.0045–0.5 nM) was incubated in triplicate in 2.0 ml of 150 mM KCl, 5 mM NaCl, 0.1 mM $CaCl_2$, 50 mM sodium phosphate (pH 7.4) with intact, unattached PC12 cells (400 μg of protein/ml) in the presence and absence of 1 μM unlabeled nitrendipine for 2 hr at room temperature. —, computer-derived fit of the data with a K_D = 0.027 nM and B_{max} = 25 fmol/mg for specific binding. (●, total binding; ○, nonspecific binding. B. Scatchard analysis of the data in A yields K_D = 0.030 nM and B_{max} = 27 fmol/mg (r = 0.96) in 150 mM KCl. The line drawn is from linear regression analysis. Also shown is [^{3}H]-(±)-nitrendipine binding (0.03–2 nM) to whole cells (△), yielding a value of 0.2 nM for the K_d and 27 fmol/mg.

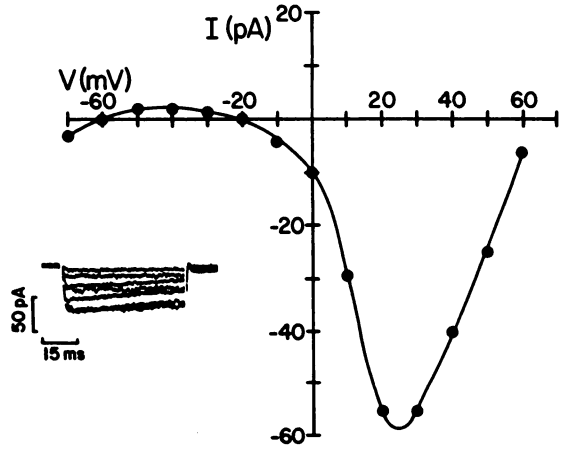

Fig. 6. The current-voltage relationship of a PC12 cell from a holding potential of −60 mV is shown in response to step depolarizations in 10-mV increments of 50 msec duration delivered at 5-sec intervals (see *inset*). The pipette contained 140 mM CsCl, 1 mM EGTA, and 10 mM HEPES (pH 7.2). The bath contained, in mM: 40 $CaCl_2$, 10 tetraethylammonium chloride, 5 4-aminopyridine, 5 KCl, 10 HEPES, 10 glucose, and 55 Tris·HCl (pH 7.4).

pH 7.4) was −11 ± 6 mV (n = 15). The binding is characterized by a single class of binding sites having an apparent dissociation constant of 0.63 ± 0.31 nM and a B_{max} equal to 55 ± 22 fmol/mg (n = 4). This corresponds to between 2000 and 4000 sites/cell based on an average measured value of 0.1 pg of protein/cell. Nonspecific binding was defined as that not displaceable by 1 μM unlabeled nitrendipine and for [^{3}H]-(±)-nitrendipine was always a high percentage of the total binding (60–85%). Numerous different attempts to lower the nonspecific binding were unsuccessful. Over the concentration range needed to define the K_D and B_{max} of [^{3}H]-(+)-PN200-110 binding, the nonspecific binding was considerably less than at the concentrations needed to define the binding constants for [^{3}H]-(±)-nitrendipine. In 150 mM Na_2HPO_4 (pH 7.4) the K_D for [^{3}H]-(+)-PN200-110 was 0.02 nM and the B_{max} was 15 fmol/mg. Incubation was carried out in 150 mM KCl, 5 mM NaCl, 0.1 mM $CaCl_2$, 50 mM MOPS (pH 7.4). The measured membrane potential under these conditions was −3 ± 2 mV (n = 10). The K_D for [^{3}H]-(+)-PN200-110 binding to intact depolarized cells is 0.044 ± 0.012 nM (n = 3). The B_{max} was 26 fmol/mg of protein. No evidence of a low affinity binding site was obtained with intact cells. A comparison of [^{3}H]-(±)-nitrendipine and [^{3}H]-(+)-PN200-110 binding shown to intact cells in the high KCl buffer is shown in Fig. 5. As can be seen, the B_{max} for [^{3}H]-(+)-PN200-110 is approximately the same as for [^{3}H]-(±)-nitrendipine binding.

Determination of the number of functional calcium channels. Calcium currents were measured in cells where a B_{max} for a nitrendipine binding of 41 fmol/mg of protein was obtained. Whole cell and single channel calcium currents were obtained in the presence of 40 mM extracellular calcium (Ca_o). In the solutions used, Na and K currents were suppressed and the remaining currents were the Ca current and a small leakage current. The Ca current could be blocked by inorganic blockers, for example, substitution of Co for Ca or addition of 1.0 mM Ni. They could also be altered by the dihydropyridines. Ca (40 mM) was used in both the whole cell and single channel experiments; this concentration was necessary to resolve the single channel current. The number of functional channels (N) per cell could be calculated from

$$I = Np_oi \quad (1)$$

where I is the whole cell current, i the single channel current, and p_o the probability that a single channel will be open. The channels are assumed to behave independently and the two parameters, p_o and i, are functions of potential. The resting potential of these cells measured immediately upon penetration was −35 to −40 mV. This fell to about −10 mV as cesium from the electrode entered the cell and blocked potassium channels. The input resistance under those conditions ranged from 10 to 50 GΩ. When the potential of the cell was held at −40 mV the holding current required was smaller than the background noise of ~5 pamp. An example of the Ca current which is slowly inactivating in these cells is shown in the *inset* of Fig. 6. The current-voltage relation for the peak current is also shown in this figure. This was obtained by applying 100-msec depolarizing voltage steps in 10-mV increments from −60 mV every 5 sec. The figure shows that the threshold for calcium current activation was about −10 mV. The nadir occurred between +20 and +30 mV. This is expected in 40 mM Ca_o as elevated calcium has been shown to shift the activation potential relation for Ca currents to more depolarized potentials (4). The peak current obtained when the cell was depolarized to +10 mV was measured in eight cells of approximately 10-μm diameter and ranged from 10 to 80 pamp. At this potential the current decayed very slowly and was essentially in a steady state. Under the same

conditions (cells exposed to 40 mM Ca_o in which resting potential averaged −40 mV), single channel calcium currents were measured when the cell-attached membrane patch was depolarized 50 mV to an estimated +10 mv. An example of single channel currents obtained in this condition is shown in Fig. 7. Single channel amplitude at this potential was obtained from the measurements of a total of 600 single channel events from two patches, each of which contained only one channel. Single channel amplitude was 0.32 pamp in one patch and 0.29 pamp in the other at this potential. The probability of a single channel opening was obtained by averaging the single channel records including the failures and dividing by the single channel amplitude. The average current during the step depolarization of the first patch, where the single channel amplitude was 0.32 pamp, was about 0.015 pamp (shown in Fig. 8). Thus, the probability of the channel being open (P_o) during the peak of the current was 0.015/0.32 = 0.05. A midrange value of whole

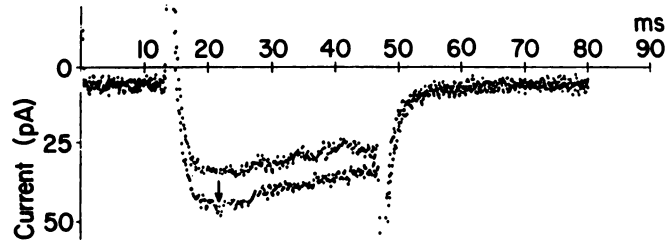

Fig. 7. The agonist effect of nitrendipine is indicated by the arrow (↓) which identifies the response to a step depolarization from −100 mV to +10 mV in the presence of 10^{-8} M nitrendipine. The response retains the shape of the smaller control response. The data are uncorrected for leakage.

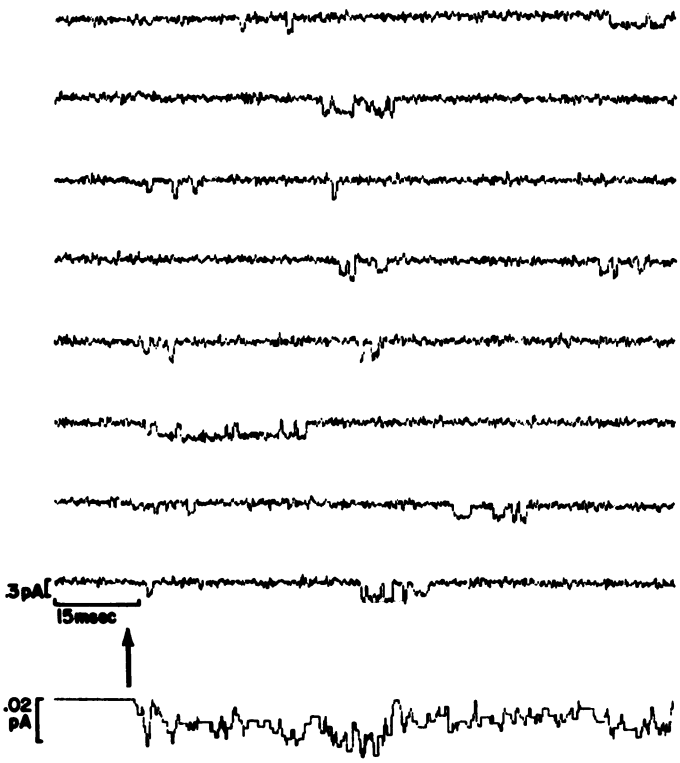

Fig. 8. The single channel currents from a cell-attached patch were filtered at 800 Hz. The test potential was +50 mV from the resting potential. The *arrow* indicates the beginning of the test pulse. The same solution described for the bath in Fig. 6 was used for the bath and the pipette in this experiment.

cell current (I) at this potential was 40 pamp. From Eq. 1 we calculate

$$40 \text{ pamp} = N\ (0.05\ (0.32)$$

$$N = 2500 \text{ channels}$$

A lower value of 10 pamp for the whole cell current would give 670 functional channels whereas an upper value of 80 pamp would give 5000 functional channels. The same calculation from data obtained in the second patch, where $P_o = 0.04$, gave a range of 860–6900 channels.

The effects of nitrendipine on whole cell calcium currents. Calcium channels may be in one of at least three states: open, closed, or inactivated. The proportion of channels in each of the states is determined by the rate constants entering and leaving each state and these rate constants are voltage dependent. Hyperpolarized potentials (more negative than −40 mV) favor the closed (resting) state. When channels are opened by depolarizing voltage steps, inactivation of a channel may follow if the depolarization is of sufficiently long duration. Inactivation may be removed by holding the membrane potential at hyperpolarized potentials. Whole cell currents were produced by depolarizing the cell for 100 msec from a holding potential of −100 mV to +10 mV while the extracellular solution contained 10 or 20 mM calcium. In these experiments the holding potential of −100 mV maximized the number of channels in the resting state and, therefore, the possibility that the drug influences the response to the depolarizing test pulse by binding to the resting state of the channel was examined. The depolarizing pulses were delivered at 5- to 10-sec intervals. The drug was added to the bathing solution in log units from 1 nM to 10 μM. Ten min at each new concentration was found to be sufficient to reach a steady state value in the magnitude of the calcium current. Upon exposure to nitrendipine at concentrations ≤ 10 nM, a small increase in calcium current occurred (Fig. 7). At higher concentrations this was followed by a decrease. The dose response curve for inhibition obtained from four preparations using the protocol described above (depolarizing steps to +10 mV from holding potential of −100 mV) is shown in the *lower curve*) of Fig. 9A. This curve illustrates the low potency of nitrendipine under these conditions. The IC_{50} was 300 nM and there was a maximum inhibition of only 33%. The effects of the drug were then examined where a portion of the calcium channels was placed in the inactivated state. The proportion of channels in the inactivated state is dependent on the holding potential and was determined from the relation of activatibility to potential ($h_\infty - V$ curve) shown in Fig. 10. To obtain this relationship a depolarizing test pulse to 0 mV is applied from holding potentials which range from −100 to −10 mV. The amplitude of the test pulse is attenuated by the proportion of channels in the inactivated state which are not available to open. The $h_\infty - V$ relationship in Fig. 10 shows that, at a holding potential of −20 mV, about 50% of the channels are not available for opening as the test pulse is reduced by 50%. At this holding potential, the dose response curve had an IC_{50} of 67 nM, and the block was about 80% complete at 10 μM (Fig. 10). This block was relieved by hyperpolarizing to 100 mV for 10 sec. This is illustrated in Fig. 9B. This example is from an experiment where a test pulse to 0 mV was applied following a 10-sec prepulse to, alternately, −100 mV and −20 mV. A 10-msec pulse to −60 mV was given between the prepulse and the test pulse to close channels

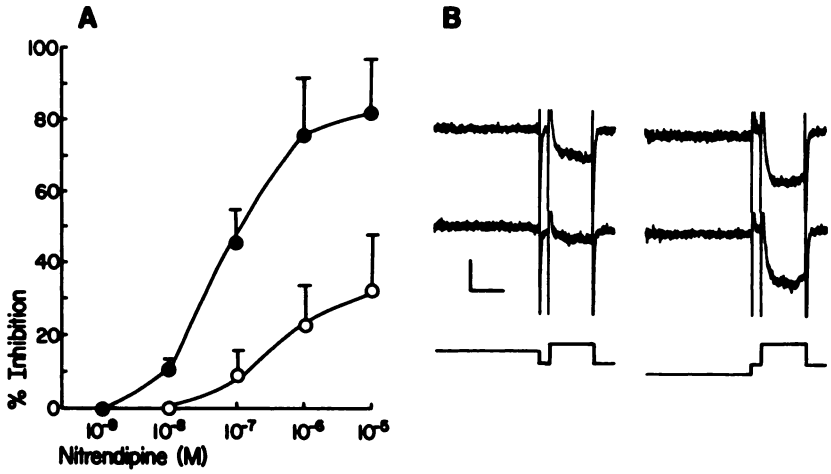

Fig. 9. A. The upper concentration curve (●) illustrates the percentage inhibition obtained in four preparations from −20 mV holding potential. The points were fit with a single-site model. % Inhibition I_{Ca} = [Drug conc.]/[Drug conc.] + K_d. The IC_{50} was 6.7×10^{-8} M. The lower curve (○) was obtained from a holding potential of −100 mV. The IC_{50} was 3×10^{-7} but maximum inhibition was only 33%. B. These current records were obtained using the protocol illustrated in the lower part of the figure. Prepulses were delivered alternately to −20 mV and −100 mV every 10 sec. The lower record was taken 6 min after the addition of 10^{-6} M nitrendipine to the bath.

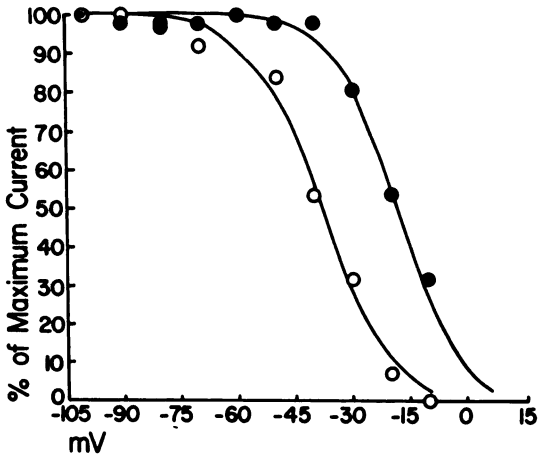

Fig. 10. The $h_\infty - V$ curve is shown for the control solution (●) and at 10^{-6} M nitrendipine (○). The prepulse potential is plotted against peak current obtained at a test pulse to +10 mV (Ca_o = 20 mM). A nonactivating or slowly inactivating component present at 10 sec was subtracted before the maximum effect was calculated. This measure of the voltage dependence of inactivation shows that it shifts to more hyperpolarized potentials in the presence of nitrendipine. The curves were fit with $(1 + \exp[(V - V_h)/k]^{-1}$ where k = 6.8.

remaining open at the end of the prepulse. In the presence of 1 μM nitrendipine there is no significant change in the amplitude of the test pulse following the −100 mV prepulse, whereas the test pulse amplitude is reduced by more than 60% following the prepulse to −20 mV. We compared the $h_\infty - V$ curves obtained in the presence and absence of 1 μM nitrendipine (Fig. 10). At $h_{1/2}$ we obtained a shift of 18–26 mV in the hyperpolarized direction (mean = 23 mV in 3 cells) in the presence of nitrendipine.

The effect of nitrendipine at 10 nM was also examined at the single channel level (Fig. 11). This cell-attached patch contained two active channels. Depolarizing pulses of 100 msec duration and 40 mV were applied. The agonist effect is evident immediately following application of nitrendipine. This was followed by the antagonist effects. At this concentration of nitrendipine the effect was predominantly on the open probability, P_o, as there was no change in the mean open time of the channel or amplitude of the single channel current. P_o first increased from 0.05 before the drug to 0.08 at the peak of the agonist effect and then decreased to 0.025 after 10 min exposure to nitrendipine. Fig. 11A illustrates the agonist and antagonist effects by the relationship between the number of openings per trace and the trace number in successive order. Fig. 11B shows examples of the single channel records of the cell patch prior to and following drug application.

Effects of (+)-PN200-110 on whole cell currents. The effect of (+)-PN200-110 was also dependent on membrane potential. There was no inhibition of current elicited by depolarizing steps to 0 mV when the holding potential was −80 mV. As with nitrendipine, the block occurred when depolarizing prepulses preceded the test pulse (Fig. 12). Long prepulses were needed (10 sec). In contrast to nitrendipine, relief of the block required 5–10 min when the prepulses of −80 mV were delivered every 20 sec. When the peak current in response to a depolarizing test pulse to 0 mV from a 10-sec prepulse to −20 mV was plotted as a function of concentration, the dose response curve in Fig. 12 was obtained. The IC_{50} was 9×10^{-9} M. From the association rate, calculated from the PC12 membrane binding studies where membrane potential was assumed to be 0 mV, at 10^{-7} M (+)-PN200-110 the drug should be bound within 1 min. We therefore examined the effect on the amplitude of the calcium current when the membrane potential was held at 0 mV for 3 min in the presence of two different concentrations of (+)-PN200-110 (5×10^{-9} and 5×10^{-8} M). The block was 51% and 85% complete. These points are not very different from those shown on the dose response curve in Fig. 12. Thus, there is a lack of correlation between the K_D from binding and the inhibitory response of calcium channels at a potential, and at a time where binding should be complete and maximal. As shown by the dose response curve in Fig. 12, a complete block was reached with higher concentrations.

Discussion

In heart cells, nitrendipine has both stimulatory and inhibitory effects (25). We have found similar effects in PC12 cells. Because the stereoisomers of nitrendipine are not available, we have not been able to determine whether these effects are separable on such a basis. As in heart cells (26, 27) the inhibitory effect is voltage dependent. A larger portion of the channels are blocked when a depolarized holding potential is used. Although a very weak block appears under a protocol which examines block to the resting state, this could alternatively reflect a small run down of the calcium current with time, independent of nitrendipine. Others have suggested that for expression of the inhibitory effect nitrendipine binds with

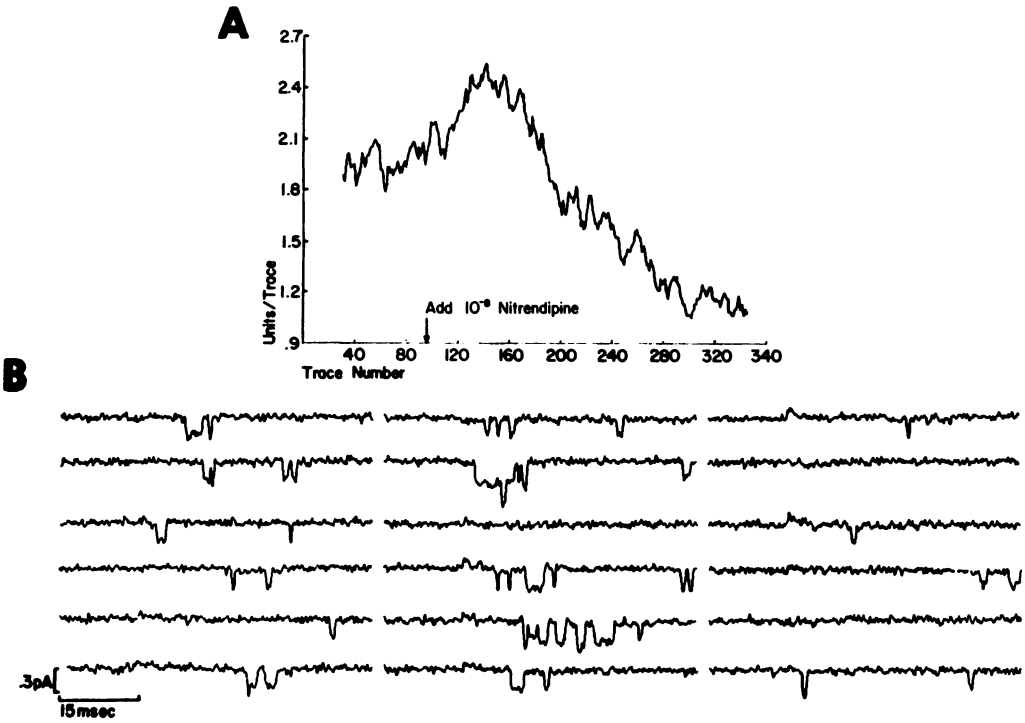

Fig. 11. A. Single channel response to nitrendipine. The number of units (openings)/record is displayed for each consecutive record for a cell-attached patch which contained two channels. At the arrow (↓) 10^{-8} M nitrendipine was added to the bathing solution. An increase in number of units per trace is followed by a decrease. B. The set on the *left* is taken from control, the *middle* from the traces at the peak of the agonist effect, and the *right* following the agonist effect. Holding potential was the resting potential. The test potential was +40 mV and was delivered every 5 sec. The patch pipette contained 40 mM Ca. The record was smoothed by averaging 20 records to obtain the first point. Each subsequent point was obtained by advancing one record and averaging 20 records beginning with that record.

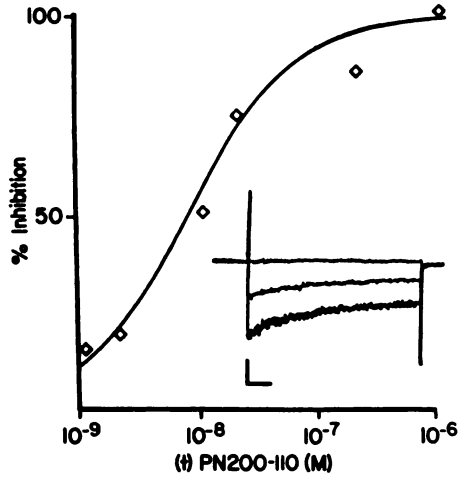

Fig. 12. A dose response curve for (+)-PN200-110 was obtained by holding at −60 mV, applying a prepulse to −20 mV for 10 sec, and measuring the current in response to a test pulse to 0 mV. Data points represent three cells. The response at each concentration stabilized after approximately 5 min when pulses were delivered every 30 sec. The responses from one cell in control solution (maximum) and in 10^{-8} M and 10^{-6} M PN200-110 are shown superimposed in the *inset* (calibration 200 msec, 50 pamp). The response was partially reversible after 5–10 min (*lower inset*) when the holding potential was −80 mV.

highest affinity to the inactivated state of the channel (26, 27). In this case, the IC_{50} of the inhibitory dose response curve will be affected by the portion of the channels in the inactivated state and will be expected to be larger than the K_D for binding to the high affinity site. The reason for this is that, in binding studies with membrane fragments, the membrane potential is zero and none of the channels are presumed to be in the resting state. In 150 mM NaCl, 50 mM Na_2HPO_4 (pH 7.4) or in 150 mM KCl, 5 mM NaCl, 0.1 mM $CaCl_2$, 50 mM MOPS (pH 7.4), in the whole cell binding experiments the cells are also depolarized. The IC_{50} or apparent K_I (K_{app}) may be estimated by assuming a modulated receptor model in which only a proportion of channels are in the inactivated state and, thus,

$$K_{app} = \frac{1}{(h/K_R) + (1 - h)K_I} \qquad (2)$$

where K_{app} is the IC_{50} obtained from a depolarized holding potential in which a fraction, h, of the channels are in the inactivated state (27). When we make this calculation assuming no block to the rested state, i.e., K_R (the dissociation constant for binding to the rested state) is very large, we obtain an average value for K_I (the dissociation constant for binding to the inactivated state) of about 30 nM. A shift in the $h_\infty - V$ curve is also predicted with a block to the inactivated state and can be used to calculate the K_I using the relationship

$$-V_h = k \ln[(1 + [\mathrm{Nit}])/K_R)/(1 + [\mathrm{Nit}])/K_I] \qquad (3)$$

where V_h is the shift in the $h_\infty(V)$ relation and is the slope factor for the relation to the inactivated state of the calcium channel (28). The −23-mV shift we obtained predicts a K_I of 29 nM which is in good agreement with the value obtained using Eq. 2. The value of 30 nM in the PC12 cells still differs from the K_D of [^{3}H]nitrendipine binding for the high affinity site binding (K_D = 0.6 nM) by 50-fold. The IC_{50} for the effect of (+)-PN200-110 was more than 200 times larger than the K_D for the same compound. A second class of calcium channels, which are activated from more negative holding potentials and which are insensitive to dihydropyridines (29, 30), was not present in the PC12 cells used in these studies and therefore cannot account for the remaining difference between the K_D and IC_{50} values. The differences between our results and those of two other laboratories, where calculations based on the modulated receptor hypothesis and which explained the discrepancy in IC_{50} and K_D, are unexplained (26, 27).

The binding constants of [^{3}H]-(±)-nitrendipine to the high affinity site in PC12 membranes obtained in our laboratory are

in close agreement with those found in other laboratories (9, 10, 32). Toll (9) also calculated from flux measurements that the unitary current was about 3×10^3 ions/sec. Taking into account a 20-fold difference in Ca^{2+} concentrations and assuming a linear relationship between current and concentration, this would be about 1/20 the value we measured directly. However, Toll's calculations (9) assumed an opening probability of 1.0, whereas in the K-depolarizing solutions he used, the membrane potential probably was close to the test potentials used in our experiments. This would give an opening probability of 0.05 for his experiments and bring his results into agreement with ours.

The agreement between the number of high affinity binding sites and the calculated number of functional Ca channels is strong evidence that the high affinity sites and the Ca channels are closely related. At the present time, we cannot discriminate between the high and low affinity [^{3}H]-(±)-nitrendipine-binding sites as the site of either the inhibitory or the stimulatory action of nitrendipine on voltage-dependent Ca channels. The presence of the stimulatory effect at doses of 1 nM and 10 nM suggests that it too is related to a high affinity site.

In addition to the high affinity site, we report the presence of a low affinity site with a K_D for nitrendipine of around 70 nM and a B_{max} of 70 pmol/mg. Litzinger and Brenneman (12) have reported the existence of a low affinity nitrendipine-binding site in spinal cord neurons. Although the dissociation constant for the low affinity site is close to the calculated K_I determined from electrophysiological studies, the number of binding sites calculated for this binding site is a thousandfold greater than the number of calcium channels determined electrophysiologically. Although one could conceivably account for this difference in terms of a large pool of nonfunctional channels, the binding of dihydropyridines to this site is not altered by antagonists such as verapamil and diltiazem.[2] Its stereoselectivity, however, has not yet been examined. The functional significance of the low affinity site is therefore unknown. It may be related to the weak tonic block of rested channels that we observed. However, the low affinity sites may include Na or K channels (33, 34). Moreover, we have recently found evidence for a low affinity nitrendipine-binding site in purified mitochondria,[3] and the low affinity site associated with crude membranes may result from mitochondrial contamination of these fractions. This possibility is currently being investigated.

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[3] K. L. Brush, M. Perez, M. J. Hawkes, D. R. Pratt, and S. L. Hamilton, submitted for publication.

Send reprint requests to: Dr. Diana L. Kunze, Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.