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Low-Affinity Binding Sites for 1,4-Dihydropyridines in Skeletal Muscle Transverse Tubule Membranes Revealed by Changes in the Fluorescence of Felodipine†

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ABSTRACT: The fluorescence changes accompanying the binding of the fluorescent calcium channel antagonist, felodipine, to transverse tubule membranes from rabbit skeletal muscle have been used to characterize low-affinity binding sites for 1,4-dihydropyridine derivatives in these preparations. In competition experiments, felodipine inhibited the high-affinity binding of (+)-[^{3}H]PN200-110 to transverse tubule membranes with an apparent K_i of 5 ± 2 nM. Binding of felodipine to additional low-affinity sites resulted in a large, saturable (K_d = 6 ± 2 μM) increase in its fluorescence which could be excited either directly (380 nm) or indirectly via energy transfer from membrane protein (290 nm). The observed fluorescence enhancement was competitively inhibited by other 1,4-dihydropyridines with inhibition constants of 3–21 μM but was unaffected by the structurally unrelated calcium channel antagonists, diltiazem and verapamil, or by Ca^{2+}, Cd^{2+}, and La^{3+}. Both high- and low-affinity binding sites appear to be localized in the transverse tubular system, since the magnitude of the observed fluorescence enhancement was higher in these membranes than in microsomal preparations and was directly proportional to the density of high-affinity sites for (+)-[^{3}H]PN200-110. Furthermore, both high- and low-affinity sites appear to be conformationally coupled since, over the same concentration range that the fluorescence changes were observed, felodipine accelerated the rate of dissociation of [^{3}H]PN200-110 previously bound to its high-affinity sites. Similar behavior has previously been reported for other 1,4-dihydropyridines [Dunn, S. M. J., & Bladen, C. (1991) *Biochemistry 30*, 5716–5721]. These results suggest that skeletal muscle transverse tubule membranes carry both high- and low-affinity binding sites for 1,4-dihydropyridines and that these sites may be important for regulation of calcium channel activity.

The 1,4-dihydropyridine (DHP)[1] class of calcium channel activators and antagonists have been widely exploited as tools in the study of the structure and function of calcium channel proteins. Radiolabeled DHPs have been shown to bind specifically and with high affinity (nanomolar K_ds) to membrane preparations from a variety of excitable tissues including smooth muscle, cardiac muscle, skeletal muscle, and brain [reviewed by Hosey and Lazdunski (1988)]. Transverse tubule

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[1] Abbreviations: DHP, 1,4-dihydropyridine; DMSO, dimethyl sulfoxide; felodipine, 4-(2,3-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid 3-ethyl 5-methyl ester; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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membranes from rabbit skeletal muscle are the richest known source of DHP binding proteins (Fosset et al., 1983; Glossmann et al., 1983), and skeletal muscle has therefore been the preferred source for molecular characterization of dihydropyridine-sensitive calcium channels [see Hosey and Lazdunski (1988)]. However, despite extensive investigation, the physiological roles of the DHP binding proteins in skeletal muscle remain to be established since calcium entry through slow voltage-gated channels is not necessary to trigger contraction [reviewed by Bean (1989); Hess, 1990]. This has led to the proposal that DHP binding proteins may play a dual role, acting both as calcium channels and as voltage sensors for excitation–contraction coupling (Rios & Brum, 1987).

In smooth muscle, a comparison of binding data with pharmacological data has shown that DHP antagonists inhibit contractility with potencies similar to their estimated affinities for binding to isolated membranes. However, anomalous behavior is found in cardiac muscle, skeletal muscle, and neuronal tissue. In these tissues, the concentrations of DHPs that are pharmacologically active are much greater than those required to saturate the high-affinity sites measured in vitro [see Triggle and Rampe (1989)]. This anomaly may be explained, at least in part, by the voltage sensitivity of DHP binding. In electrophysiological experiments using cardiac preparations, it was found that the ability of several DHPs to inhibit calcium currents was increased under depolarized conditions (Bean, 1984; Sanguinetti & Kass, 1984). DHPs may therefore bind more tightly to depolarized preparations, such as isolated membranes. An alternative explanation for the observed quantitative discrepancies is that DHP binding proteins carry multiple binding sites and that the effects of DHPs on function may be due to their binding to distinct low-affinity sites. In this respect, we have recently reported that, in addition to the high-affinity binding sites that are readily measured in direct radiolabeled binding studies, there are additional low-affinity sites for DHPs in skeletal muscle transverse tubule membranes (Dunn & Bladen, 1991). These latter sites were revealed by the ability of micromolar concentrations of several DHPs to accelerate the rate of dissociation of [^{3}H]PN200-110 from its high-affinity sites.

In the present study, the presence of low-affinity binding sites for DHPs in skeletal muscle transverse tubule membranes has been further investigated. The binding of micromolar concentrations of the calcium channel antagonist, felodipine, is accompanied by a specific and saturable increase in its fluorescence which may be excited either directly or indirectly via energy transfer from the transverse tubule membrane protein. The fluorescence enhancement is competitively inhibited by other DHPs but not by other calcium channel modulators. Direct evidence is thus provided for the presence of specific low-affinity sites for 1,4-dihydropyridines in transverse tubule membranes that may be important in calcium channel function.

Materials and Methods

Materials. Felodipine was a kind gift of Dr. M. Nordlander (A. B. Hässle, Mölndal, Sweden). (±)Bay K8644, (±)nitrendipine, and (±)nimodipine were generously provided by Dr. A. Scriabine (Miles Laboratories, New Haven, CT). The stereoisomers (+)PN200-110 and (−)PN200-110 were generously provided by Drs. D. Römer and E. Rissi (Sandoz Ltd., Basle, Switzerland). Nifedipine, verapamil, and diltiazem were from Sigma Chemical Co. (St. Louis, MO). [^{3}H]PN200-110 was from Du Pont, New England Nuclear.

Preparation of Skeletal Muscle Membranes. Microsomal membranes and purified transverse tubule membrane vesicles were prepared from the back and hind leg muscles of small (2–3 lb) New Zealand white rabbits as previously described (Dunn, 1989). Triads were prepared as described by Mitchell et al. (1983). Protein concentrations were measured by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA).

Fluorescence Experiments. Equilibrium fluorescence experiments were carried out using a Perkin-Elmer MPF4 fluorometer thermostated at 25 °C. Unless otherwise indicated, the buffer used in each experiment was 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4. In the fluorescence titrations, 2 mL of buffer or transverse tubule membranes (5 μg/mL) was added to quartz cuvettes in the case of energy transfer experiments or, when direct felodipine excitation was used, to disposable polystyrene cuvettes (Sarstedt Canada, St. Laurent, Quebec, Canada). Aliquots (1 μL) of concentrated felodipine solutions in DMSO were added to the samples which were continuously stirred. Fluorescence readings were recorded immediately, and the excitation shutter was closed between readings to minimize felodipine photolysis. In the competition experiments, 2 mL of transverse tubule membranes was incubated with 5 μM felodipine for 15 min prior to the addition of small (1-μL) aliquots of concentrated solutions of DHPs also prepared in DMSO. In a control sample, 5 μM felodipine was titrated with DHPs in the absence of transverse tubule membranes, and the results of these titrations were used to correct for spectral interference occurring at high concentrations of competing DHPs. The final concentration of DMSO did not exceed 1%.

Stopped-Flow Measurements of Felodipine Binding to Transverse Tubule Membranes. Kinetic experiments were carried out using an Applied Photophysics Ltd. (Leatherhead, England) SF.17MV microvolume stopped-flow spectrofluorometer thermostated at 25 °C. The excitation wavelength was either 290 or 380 nm as indicated in the text, and fluorescence emission was monitored using a GG420 filter cutoff filter (Melles Griot, Irvine CA) which excludes light below 400 nm.

Binding of (+)-[^{3}H]PN200-110 to Membrane Preparations. The binding of (+)-[^{3}H]PN200-110 at equilibrium and in kinetic experiments was measured in filtration assays as previously described (Dunn & Bladen, 1991). Briefly, membranes (0.002–0.05 mg/mL) were incubated with (+)-[^{3}H]PN200-110 at room temperature (23 ± 2 °C) and, at the appropriate time, an aliquot was removed and filtered under vacuum through Whatman GF/C filters using a Hoefer filtration manifold. The filters were immediately washed with two 5-mL aliquots of ice-cold buffer. After drying and the addition of 5 mL of ACS (Amersham Canada Ltd., Oakville, Ontario, Canada) scintillation fluid, the filters were counted for ^{3}H. In the dissociation experiments, 1 nM (+)-[^{3}H]PN200-110 was first incubated with microsomal membranes (50 μg/mL) for 40 min at room temperature. Dissociation was initiated by the addition of the appropriate concentration of felodipine as indicated in the text and filtering of 0.5-mL aliquots at the appropriate times followed by washing and counting for ^{3}H as above.

Data Analysis. Kinetic data were analyzed using the Applied Photophysics Ltd. kinetic software package and the models described in the text. Other data were analyzed by nonlinear regression techniques using the algorithm of Marquardt adapted from Bevington (1969).

Results

High-Affinity Binding of Felodipine Measured by Displacement of (+)-[^{3}H]PN200-110. The DHP calcium channel antagonist, (+)-[^{3}H]PN200-110 binds with high affinity (K_d

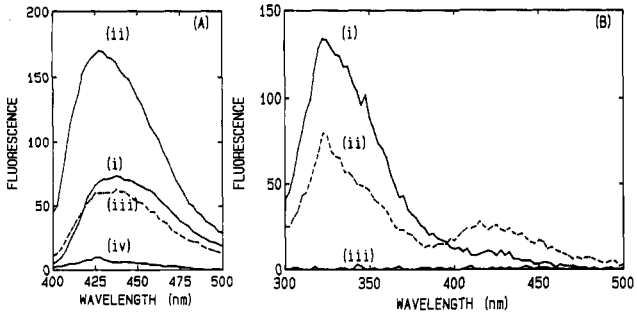

FIGURE 1: Enhancement of felodipine fluorescence upon binding to transverse tubule membranes. (A) Fluorescence emission spectrum of felodipine (5 μM in 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4) before (i) and after (ii) the addition of transverse tubule membranes to a final concentration of 5 μg/mL. The excitation wavelength was 370 nm. Spectrum iii was recorded after the addition of nitrendipine (50 μM final concentration) to the transverse tubule–felodipine complex shown in spectrum ii, and spectrum iv shows light scattering by transverse tubule membranes (5 μg/mL) in the absence of felodipine. (B) Excitation of felodipine fluorescence via energy transfer using an excitation wavelength of 290 nm. Spectrum i shows transverse tubule membrane (5 μg/mL) fluorescence; spectrum ii was recorded after the addition of 5 μM felodipine resulting in a quench of protein fluorescence at about 330 nm and the appearance of felodipine fluorescence emission at about 430 nm; spectrum iii shows 5 μM felodipine alone to demonstrate that felodipine fluorescence cannot be directly excited at 290 nm.

= 0.30 ± 0.05 nM) to membrane preparations from rabbit skeletal muscle (Dunn, 1989). Competition experiments have been used to investigate the affinity of felodipine for these high-affinity DHP binding sites. Felodipine was found to displace bound (+)-[^{3}H]PN200-110 (data not shown), in an apparently simple competitive manner with a K_i of 5 ± 2 nM (n = 7).

Effect of Irradiation of DHPs of High-Affinity Binding. Since DHPs are known to be photosensitive (Eisner & Kuthan, 1972) and, in the present study, optical techniques have been used to investigate DHP binding, it was important to establish that the DHPs were not photolyzed under the conditions of the fluorescence experiments. Solutions of felodipine, nitrendipine, and Bay K8644 were therefore prepared at final concentrations of 1 and 10 μM in quartz cuvettes, and these were placed in the fluorometer sample compartment and were continuously irradiated for 10 min at either 290 or 380 nm under conditions identical to those used in the fluorescence experiments. The irradiated samples and controls that had been kept in the dark were then tested for their ability to displace (+)-[^{3}H]PN200-110 from its high-affinity binding sites. In these experiments, it was found that this irradiation had no significant effect on the competition curves, and thus, it is assumed in the discussion below that photolysis did not make a significant contribution to the experimental results.

Fluorescence of Felodipine and Evidence for Low-Affinity Binding Sites in Transverse Tubule Membranes. Felodipine exhibits broad fluorescence excitation and emission maxima at 380 nm (not shown) and 430 nm (Figure 1A), respectively. Upon the addition of transverse tubule membranes (5 μg/mL final concentration) to 5 μM felodipine, the fluorescence of felodipine was enhanced by approximately 2-fold, and this enhancement was reversed by the addition of 50 μM nitrendipine (Figure 1A). Complex formation between transverse tubule membranes and felodipine could also be monitored by energy transfer fluorescence as shown in Figure 1B. Transverse tubule membranes are themselves highly fluorescent, and when excited at 290 nm, the fluorescence emission spectrum shows a maximum at about 330 nm, typical of the fluorescence spectra reported for other membrane-bound proteins. The addition of felodipine (5 μM final concentration) quenched this fluorescence, and a new maximum appeared in the emission spectrum at 400–450 nm. This behavior is characteristic of activation of felodipine fluorescence by energy transfer from membrane protein. Since energy transfer occurs only over limited distances (<70 Å), this places restrictions on the distance of the felodipine fluorophore from the protein donor [see Stryer (1978)].

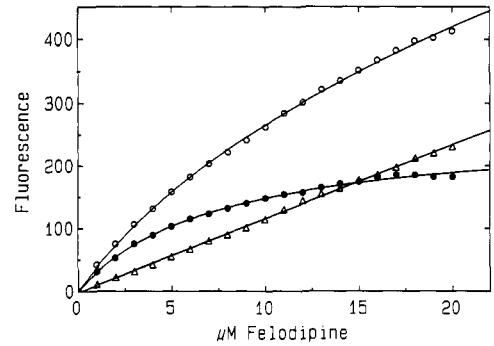

FIGURE 2: Fluorescence titration of transverse tubule membranes by felodipine. Transverse tubule membranes (5 μg/mL in a total volume of 2 mL) in 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4 (○) or buffer alone (△) were continuously stirred and titrated with 1-μL aliquots of stock felodipine in DMSO to give the indicated final concentrations. The fluorescence was recorded using excitation and emission wavelengths of 370 and 430 nm, respectively. The solid symbols show the difference between the two curves, and the solid line is calculated using the best-fit parameters obtained from fitting by a simple binding isotherm, $Fl = Fl_{max}[L]/(K_d + [L])$ where Fl is the observed fluorescence, Fl_{max} is the maximum specific fluorescence enhancement, and [L] is the felodipine concentration. Best-fit values for Fl_{max} and K_d were 261 (arbitrary units) and 7.6 μM, respectively.

The felodipine concentration dependence of the fluorescence enhancement has been measured in equilibrium fluorescence titrations, an example of which is illustrated in Figure 2. It should be noted that, in these experiments, the maximal concentration of felodipine that can be attained experimentally is about 20 μM due to its limited aqueous solubility. However, it is clear from the data shown in Figure 2 that the fluorescence enhancement shows saturability and indicates the presence of a population of low-affinity binding sites having a K_d of 6 ± 2 μM (n = 7). Similar results have been obtained using excitation of felodipine fluorescence via energy transfer although, as expected, the magnitude of the fluorescence enhancement was much reduced.

Effects of Other 1,4-Dihydropyridines on Felodipine Fluorescence. When the felodipine–transverse tubule complex was titrated with increasing concentrations of other DHPs, the fluorescence enhancement was reversed in an apparently competitive manner as illustrated in Figure 3. All DHPs examined were able to compete for the low-affinity felodipine binding with K_i values ranging from 3 to 21 μM, with the agonist (±)Bay K8644 being the most potent. In addition to the data shown in Figure 3, K_i values have also been measured for nifedipine (21 μM) and (±)nimodipine (15 μM). In these experiments, the results of parallel titrations of free felodipine were used to correct for interference due to absorbance of some of the DHPs at the excitation wavelength used (380 nm). In the case of (±)Bay K8644, which shows the greatest absorbance in this region, identical results have been obtained in fluorescence titrations carried out using excitation and emission wavelengths of 350 and 480 nm, i.e., far from the felodipine maxima but at wavelengths at which there is little spectral interference from Bay K8644.

Effect of Other Calcium Channel Ligands and Cations on Felodipine Binding. The DHP binding protein also carries

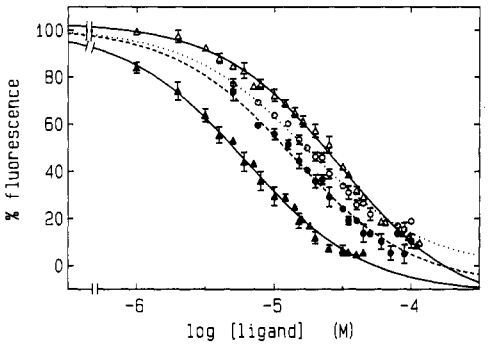

FIGURE 3: Effect of other 1,4-dihydropyridines on the enhancement of felodipine fluorescence. Transverse tubule membranes (5 μg/mL) were incubated with 5 μM felodipine for 15 min at 25 °C prior to titration by increasing amounts of (±)Bay K8644 (▲), (+)PN200-110 (●), (−)PN200-110 (○), or (±)nitrendipine (△) from concentrated stock solutions in DMSO. At each ligand concentration the fluorescence was recorded using excitation and emission wavelengths of 370 and 430 nm, respectively. Data were corrected for nonspecific effects due to absorbance by the competing DHPs using the results of parallel titrations of felodipine alone. Data shown are average values (±sd) of at least three determinations. Solid lines are calculated using the best-fit parameters obtained from fitting by the model, bound = bound/(1 + [I]/IC_{50}) where [I] is the competing ligand concentration. K_i values were calculated from the relationship, $K_i = IC_{50}/(1 + [L]/K_d)$ where [L] is the felodipine concentration (5 μM) and the K_d is 6 μM as described in the text. Best fit values for K_i were 3.3 μM (▲), 7.2 μM (●), 9.0 μM (○), and 15.8 μM (△).

binding sites for structurally unrelated calcium channel blockers, including benzothiazepines such as diltiazem and phenylalkylamines such as verapamil. At concentrations up to 1 mM, these ligands did not affect the fluorescence of the felodipine–transverse tubule membrane complex. The fluorescence enhancement upon complex formation also had no obvious Ca^{2+} dependence. Fluorescence titrations by felodipine were identical in buffer containing 1 mM $CaCl_2$ or 1 mM EDTA. The high-affinity binding of DHPs to skeletal muscle membranes has recently been shown to be dependent on extracellular calcium (Ebata et al., 1990). This corresponds to intravesicular calcium in the case of transverse tubule vesicles which are oriented inside out [see Dunn (1989)]. In order to investigate whether the low-affinity binding is also modulated by intravesicular calcium, the transverse tubule vesicles were first loaded with 1 mM EDTA by cycles of freezing and thawing and fluorescence titrations were carried out in the presence of 1 mM EDTA. This also did not affect the magnitude of the specific fluorescence enhancement or the estimated affinity for felodipine. In other experiments, neither Cd^{2+} nor La^{3+}, at concentrations up to 10 mM, affected the fluorescence of the felodipine–transverse tubule complex.

Kinetics of Felodipine Binding to Transverse Tubule Membranes. In a preliminary study of the mechanism of felodipine binding, the kinetics of association and dissociation have been investigated in stopped-flow fluorescence experiments (Figure 4). At all concentrations of felodipine (1–10 μM) examined, the kinetics of association showed systematic deviation from a single-exponential process. The kinetics could, however, be adequately described by a two-exponential model as shown in Figure 4A. At 5 μM felodipine, two processes with apparent rate constants of approximately 50 s^{-1} and 5 s^{-1} were observed with approximately 65% of the measured fluorescence enhancement occurring in the faster phase. The observed kinetics were independent of whether excitation was by direct means or by energy transfer from the protein (Figure 4A). The rate of the slower process showed no obvious felodipine concentration dependence, but the rate of the faster phase increased in an approximately hyperbolic fashion from

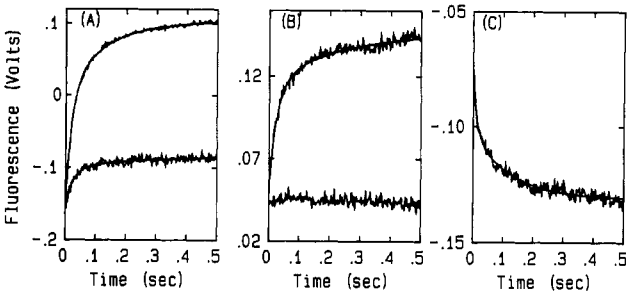

FIGURE 4: Kinetics of felodipine binding measured in stopped-flow experiments. Solid lines in each case were calculated using the best-fit parameters obtained by data fitting by a double-exponential model, $Fl(t) = A_1 \exp(-k1t) + A_2 \exp(-k_2t) + A_0$ where $Fl(t)$ is the fluorescence level at time t; A_1 and A_2 represent the amplitudes of the two phases; k_1 and k_2 are their corresponding rate constants; and A_0 is the equilibrium fluorescence level. (A) Association of felodipine (5 μM) with 5 μg/mL transverse tubule membranes using direct fluorescence excitation at 370 nm (upper trace) or by energy transfer at 290 nm (lower trace). Best-fit parameters for the upper trace were A_1 = 167 mV, k_1 = 48.9 s^{-1}, A_2 = 102 mV, and k_2 = 8.0 s^{-1} and for the lower trace were A_1 = 57 mV, k_1 = 44 s^{-1}, A_2 = 29 mV, and k_2 = 5.3 s^{-1}. (B) Block by nitrendipine. In the upper trace, 1 μM felodipine was reacted with 5 μg/mL transverse tubule membranes and the fluorescence was recorded using an excitation wavelength of 370 nm. The best-fit parameters were A_1 = 65 mV, k_1 = 39 s^{-1}, A_2 = 32 mV, and k_2 = 4.7 s^{-1}. In the lower trace, membranes were preincubated with 50 μM nitrendipine before being mixed with 50 μM nitrendipine and 1 μM felodipine (final concentrations), showing the complete block of the felodipine fluorescence enhancement. (C) Displacement of felodipine by nitrendipine. Felodipine (1 μM) was preincubated with transverse tubule membranes for 10 min at 25 °C before being mixed with an equal volume of buffer containing 1 μM felodipine and 100 μM nitrendipine. The solid line is calculated using the best-fit parameters A_1 = −32 mV, k_1 = 90 s^{-1}, A_2 = −29 mV, and k_2 = 8 s^{-1}.

35 ± 5 s^{-1} at 1 μM to 65 ± 5 s^{-1} at 10 μM. Unfortunately, rigorous examination of the kinetic mechanism is, at this time, precluded by the restrictions imposed by the limited solubility of felodipine.

The kinetic data in Figure 4B show that the fluorescence enhancement occurring upon mixing of transverse tubule membranes with felodipine is abolished by preincubation of the membranes with excess nitrendipine, and this is consistent with the equilibrium results. When the complex formed between 1 μM felodipine and transverse tubule membranes was mixed with 50 μM nitrendipine, a rapid fluorescence quench was observed which is likely due to the dissociation of felodipine and its replacement by the nonfluorescent ligand. The kinetics in this case were also perceptibly biphasic with measured rate constants of 90 s^{-1} and 8 s^{-1}. However, these values should be considered only as estimates and not as true dissociation rate constants. This is unavoidable since, due to the limited solubility of the competing DHP, it is not possible to use a sufficient excess of the competing ligand to fulfill the criteria required for measurement of dissociation constants in such experiments.

Transverse Tubule Localization of Low-Affinity DHP Binding Sites. Although the experiments described above show that transverse tubule membrane preparations carry low-affinity binding sites for DHPs, it was not possible to exclude the possibility that the observed binding was to components of a contaminating membrane population. The binding of felodipine to other membrane preparations having different levels of enrichment in high-affinity binding sites for (+)-[^{3}H]PN200-110, i.e., microsomal, triad, and transverse tubule membranes, has therefore been investigated. Although all these preparations showed a saturable fluorescence enhancement upon felodipine binding (with similar K_d values of about

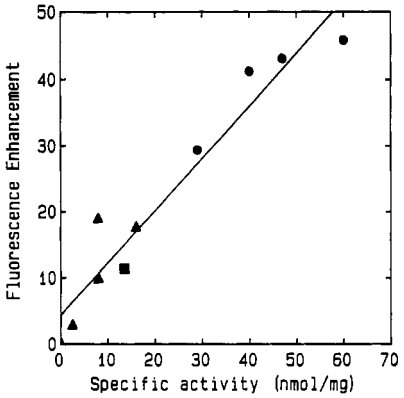

FIGURE 5: Correlation of the maximum specific fluorescence enhancement accompanying felodipine binding to muscle membrane preparations with the density of high-affinity binding sites for DHPs in the membrane. Data shown represent transverse tubule (●), triad (■), and microsomal (▲) membranes prepared as described in Materials and Methods. Membranes were assayed for (+)-[^{3}H]-PN200-110 binding in equilibrium filtration experiments and for the enhancement of felodipine fluorescence using the same procedure as in the legend to Figure 2. The vertical axis shows Fl_{max} obtained by model fitting as in Figure 2 and represents the average of duplicate determinations. The horizontal axis shows the specific activity of the membrane preparations in terms of nanomoles of (+)-[^{3}H]PN200-110 binding per milligram of protein.

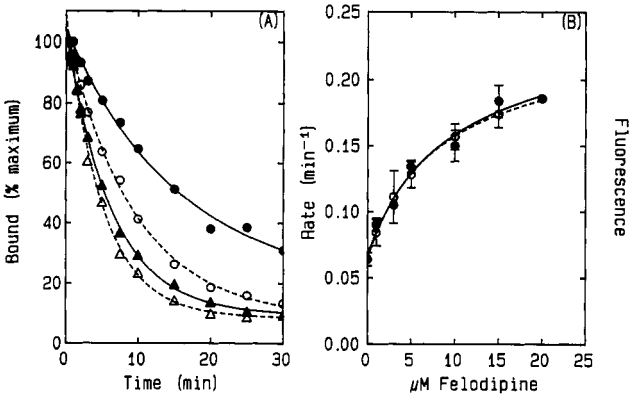

FIGURE 6: Effect of felodipine on the rate of dissociation of (+)-[^{3}H]PN200-110 from its high-affinity sites. (A) Muscle membranes (50 μg/mL) in 25 mM Hepes–Tris, 1 mM $CaCl_2$, pH 7.4 were incubated with 1 nM (+)-[^{3}H]PN200-110 for 40 min at room temperature prior to initiation of dissociation by 20-fold dilution into buffer (●) or by the addition of felodipine to a final concentration of 3 μM (○), 10 μM (▲), or 15 μM (△). Lines are calculated from the best-fit parameters obtained by fitting by a single-exponential model giving rate constants of 0.064 (●), 0.106 (○), 0.158 (▲), and 0.188 (△) min^{-1}. (B) Effect of felodipine concentration on the rate of dissociation of (+)-[^{3}H]PN200-110. Data (●) were obtained from experiments as in (A), and each point represents the average of at least two determinations. For comparison, the specific fluorescence enhancement upon the binding of felodipine to transverse tubule membranes is shown in open symbols (average of at least three determinations). The fluorescence data have been normalized to the rate data using limiting values at 0 to 20 μM felodipine.

6 μM), the magnitude of the specific enhancement was considerably greater in the transverse tubule membranes. Figure 5 shows that the magnitude of the maximum fluorescence enhancement was directly correlated with the density of high-affinity (+)-[^{3}H]PN200-110 binding sites. Although such quantitation of fluorescence is open to different interpretations, the excellent correlation provides strong circumstantial evidence that the high- and low-affinity DHP binding sites are colocalized in the transverse tubular system.

Identification of Low-Affinity Binding Sites for Felodipine by Acceleration of (+)-[^{3}*H*]*PN200-110 Dissociation Kinetics.* Felodipine has previously been shown to bind with micromolar affinity to calmodulin and to inhibit several calmodulin-dependent enzymes (see below). It was, therefore, reasonable to question whether the binding observed in the present study is due to binding to the voltage-gated calcium channel which also binds DHPs with high affinity or to some other unrelated protein present in transverse tubule membranes. The relationship between high- and low-affinity binding sites has therefore been investigated by studying the kinetics of dissociation of (+)-[^{3}H]PN200-110. As we have previously reported for other DHPs (Dunn & Bladen, 1991), micromolar concentrations of felodipine accelerated the dissociation of the radiolabeled derivative from its high-affinity sites to which it was previously bound (Figure 6A). If it assumed that dissociation follows single-exponential kinetics [although this is an approximation only, see Dunn and Bladen (1991)] the dose dependence of this phenomenon may be estimated and is illustrated in Figure 6B. Clearly, there is excellent agreement between the concentrations of felodipine that affect dissociation from the high-affinity binding sites and the fluorescence changes that accompany its binding to the low-affinity sites. This suggests not only that the same binding phenomenon is being measured using the two different techniques but also that there is some interaction between high- and low-affinity sites and that they may reside on the same protein complex.

Discussion

The predominant calcium channels in skeletal muscle are sensitive to 1,4-dihydropyridine agonists and antagonists [reviewed by Bean (1989); Hess, 1990], and both calcium channels (Almers et al., 1981) and high-affinity DHP binding proteins (Fosset et al., 1983) are enriched in the transverse tubule system. Despite extensive investigation, there are, however, many unanswered questions relating to the mechanism of action fo the DHPs, and both the number of binding sites and the functional significance of their occupancy remain to be established [see Triggle and Rampe (1989)]. The molecular basis for the discrepancy of several orders of magnitude between the concentrations of DHPs that affect functional properties such as charge movements (Rios & Brum, 1987), contractures (Eisenberg et al., 1983; McCleskey, 1985), or calcium flux responses (Dunn, 1989) and those that saturate the high-affinity sites measured in membrane preparations also remains to be elucidated. As previously discussed (Dunn & Bladen, 1991), one possible explanation for this quantitative discrepancy is that calcium channels carry multiple binding sites having different affinities and occupancy of these sites lead to different responses. In studies of radiolabeled DHP binding to cardiac muscle membranes, sites of low affinity (K_d of 25–240 nM) and high capacity (B_{max} of 8–35 pmol/mg) have been identified (Bellemann et al., 1981; Kennesey et al., 1984; Ruth et al., 1985; Rogart et al., 1986; Ferry et al., 1987). However, low-affinity DHP binding sites have previously been thought to be virtually absent in skeletal muscle transverse tubule membranes [see Ferry et al. (1987)].

The fluorescence of felodipine has previously been used to characterize its interaction with calmodulin (Johnson, 1984; Johnson et al., 1986), and this first suggested its usefulness to probe DHP binding sites in skeletal muscle membranes. In previous experiments, micromolar concentrations of DHPs were shown to accelerate the rate of dissociation of (+)-[^{3}H]PN200-110 from its high-affinity sites (Dunn & Bladen, 1991) and it was suggested that this was due to occupancy of distinct low-affinity sites which induced a conformational change in the DHP binding protein. In these experiments, it was not, however, possible to conclusively demonstrate the presence of a specific low affinity site i.e. one showing sat-

urability. Such evidence has now been obtained from studying the fluorescence changes of felodipine which accompany its binding to transverse tubule membranes. Although there was no measurable fluorescence change upon binding of felodipine to the high-affinity sites that have been identified in competition studies using radiolabeled PN200-110, there was a large, saturable increase in fluorescence at higher concentrations, suggesting the presence of low-affinity sites having a K_d of approximately 6 μM. This binding appears to DHP specific, and micromolar concentrations of several DHPs have been shown to compete for these sites. The other calcium channel ligands, diltiazem and verapamil, did not affect the observed fluorescence changes which were also unaltered in the presence of Ca^{2+}, Cd^{2+}, or La^{3+}.

Felodipine is a potent vasodilator which selectively inhibits vascular smooth muscle in concentrations that lack significant effect on cardiac muscle (Ljung, 1985). Although its principle site of action is believed to be voltage-gated calcium channels, felodipine, in the micromolar range, has also been shown to interact with calmodulin (Boström et al., 1981; Johnson, 1983) and to inhibit several calmodulin-dependent enzymes (Walsh et al., 1988). Futhermore, in photoaffinity labeling studies, [^{3}H]felodipine has been shown to label a cytosolic protein of an apparent molecular weight of 62K in vascular smooth muscle (Nilsson et al., 1987) but not in the liver or cardiac or skeletal muscle (Nilsson et al., 1989). Binding sites with micromolar affinities for other DHPs have also been identified in mitochondrial preparations (Zernig & Glossmann, 1988) which may be associated with peripheral benzodiazepine receptors (Cantor et al., 1984) or Na^+–Ca^{2+} exchangers (Vaghy et al., 1982). Other low-affinity DHP binding proteins include the nucleoside transporter (Striessnig et al., 1985), Na^+/K^+-ATPase (Pan & Janis, 1984), calmodulin-dependent cyclic AMP phosphodiesterase (Schaechtele et al., 1987), and the multidrug-resistance related P-glycoprotein (Cornwell et al., 1987).

In view of the above studies demonstrating a multiplicity of potential binding sites, it remains a possibility that the observed binding of felodipine to transverse tubule membranes is to a protein unrelated to calcium channels. In these experiments, binding to mitochondrial proteins or to soluble enzymes such as cyclic AMP phosphodiesterase would appear to be excluded since the fluorescence changes occurring on felodipine binding were much greater using highly purified transverse tubule membranes. The possibility that the observed fluorescence changes are due to binding to calmodulin itself is unlikely since the interaction of felodipine with transverse tubule membranes did not display the dependence on calcium ions which is characteristic of its binding to calmodulin (Böstrom et al., 1981).

Two observations suggest that the low-affinity DHP binding sites are located either on the same protein complex that binds DHPs with high affinity or on a protein with which this complex interacts: (1) the direct correlation between the magnitude of the fluorescence enhancement and the density of high-affinity DHP binding sites suggests that the two classes of binding sites are colocalized in the transverse tubule system; (2) the excellent agreement between the dose dependence of the fluorescence enhancement and the ability of felodipine to accelerate the rate of dissociation of (+)-[^{3}H]PN200-110 from its high-affinity sites suggests that the two classes of sites are conformationally linked. Assignment of the low-affinity binding sites to the high-affinity DHP binding protein as distinct from an associated protein will require identification of these sites in the purified protein. Preliminary attempts have failed to identify these sites in purified preparations, perhaps indicating that they are lost upon purification. However, recent observations of the greater heat stability and lower protease susceptibility of the low-affinity sites compared to the high-affinity sites (unpublished observations) suggest that the former sites may be located in a protected region, perhaps in an intramembranous location, a possibility which is supported by the hydrophobicity of DHPs. Thus, reconstitution of the purified protein may be necessary to recover the sites, although these experiments are complicated by the large nonspecific and nonsaturable increase in felodipine fluorescence as it partitions into artificial phospholipid vesicles (unpublished observations).

In the fluorescence competition experiments, it was somewhat surprising that the two stereoisomers, (+)- and (−)-PN200-110, were about equipotent in reversing the fluorescence enhancement produced by felodipine binding. In studies of (+)-[^{3}H]PN200-110 dissociation, it was previously shown that the (−) isomer (which also binds less tightly to the high-affinity DHP sites) was ineffective in accelerating the dissociation rate, suggesting that the low-affinity binding is stereospecific. The present results show that this is not the case, and it must be concluded that the isomers bind similarly but differ in their ability to produce the protein conformational change that affects the mode of DHP binding to the high-affinity site(s).

A preliminary study of the kinetics of felodipine binding has been undertaken and has demonstrated that the fluorescence changes accompanying felodipine binding to transverse tubule membranes are both rapid and reversible. The association kinetics were biphasic, and both phases were blocked by preincubation of the membranes with nitrendipine. Identical reaction profiles were observed whether fluorescence excitation was by direct felodipine excitation or by energy transfer, suggesting that both phases arise from binding to protein rather than from a nonspecific partitioning into the membrane phase. Detailed analyses of the binding kinetics are presently being undertaken.

A functional significance of low-affinity DHP binding sites has been inferred in several previous studies. Brown et al. (1986) studied the effects of DHP agonists and antagonists on whole cell and single-channel currents in guinea pig ventricular cells. The effects of nitrendipine and Bay K8644 were either monophasic or biphasic depending on membrane potential. These results were attributed to the presence of high- (nanomolar) and low-affinity (micromolar) sites, with the low-affinity sites mediating stimulatory effects due to prolonged openings and the high-affinity sites being either stimulatory or inhibitory depending on membrane potential. In skeletal muscle, occupancy of low-affinity sites has also been correlated with effects on function. Recently, Ohkusa et al. (1991) reported that the effects of DHP antagonists on depolarization induced release of Ca^{2+} from transverse tubule vesicles paralleled binding to low-affinity sites (K_d of approximately 1.5 μM) but that there was no correlation with the high-affinity binding.

In conclusion, the results described above provide direct evidence for the presence of low-affinity binding sites for felodipine and other DHP calcium channel ligands in skeletal muscle transverse tubule membranes. Although the physiological relevance of multiple DHP binding sites remains to be established, it has previously been demonstrated that in vivo felodipine accumulates in cells and intracellular concentrations of approximately 15 μM may be achieved [see Walsh et al. (1988)]. Thus, intracellular DHP concentrations may be

sufficiently high to occupy low-affinity binding sites and modulate calcium channel function.

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