Kinetics of Binding of Dihydropyridine Calcium Channel Ligands to Skeletal Muscle Membranes: Evidence for Low-Affinity Sites and for the Involvement of G Proteins†

Susan M. J. Dunn* and Christopher Bladen
Department of Pharmacology, Faculty of Medicine, The University of Alberta, Edmonton, Alberta, Canada T6G 2H7
Received August 8, 1990; Revised Manuscript Received March 13, 1991

ABSTRACT: Detailed kinetic studies of the binding of the calcium channel antagonist (+)-[^{3}H]PN200-110 to membrane preparations from rabbit skeletal muscle have demonstrated that, in addition to the high-affinity sites (K_d = 0.30 ± 0.05 nM) that are readily measured in equilibrium and kinetic experiments, there are also dihydropyridine binding sites with much lower affinities. These sites were detected by the ability of micromolar concentrations of several dihydropyridines to accelerate the rate of dissociation of (+)-[^{3}H]-PN200-110 from its high-affinity sites. The observed increase in rate was dependent on the concentration of competing ligand, and half-maximal effects occurred at approximately 10 μM for the agonist (±)-Bay K8644 and for the antagonists nifedipine, (±)-nitrendipine, and (+)-PN200-110. The low-affinity sites appear to be stereospecific since (−)-PN200-110 (1–200 μM) did not affect the dissociation rate. The possible involvement of guanine nucleotide binding proteins in dihydropyridine binding has been investigated by studying the effects of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) on binding parameters. At a concentration of 10 μM, neither GTPγS nor GDPβS significantly affected the binding of dihydropyridines to their high-affinity sites. GTPγS did, however, increase the ability of (±)-Bay K8644, but not of (±)-nitrendipine, to accelerate the rate of dissociation of tightly bound (+)-[^{3}H]PN200-110. GDPβS did not affect the dose dependence of either the agonist or the antagonist. These results suggest that skeletal muscle dihydropyridine receptors have low-affinity binding sites that may be involved in the regulation of calcium channel function and that activation of a guanine nucleotide binding protein may modulate the binding of agonists but not of antagonists to these sites.

The predominant calcium channels in skeletal muscle are sensitive to 1,4-dihydropyridines (DHPs)[1] and have been referred to as the "L"-type or "slow" calcium channels [reviewed by Bean (1989) and Hess (1990)]. In radiolabeled form, the DHPs have been extensively used to study the properties of L channels [reviewed by Hosey and Lazdunski (1988)]. DHP binding proteins are found in the transverse tubular membrane system of rabbit skeletal muscle at a density 50–100 times higher than in any other tissue (Fosset et al., 1983; Glossmann et al., 1983), and skeletal muscle has therefore been the preferred source for purification of the putative calcium channel [see Hosey and Lazdunski (1988)]. The purified protein has been reconstituted into phospholipid vesicles (Curtis & Catterall, 1986) and planar bilayers (Flockerzi et al., 1986; Smith et al., 1987) and has been shown to form a functional voltage-dependent calcium channel. However, the physiological functions of skeletal muscle calcium channels remain obscure since the influx of extracellular calcium through these channels is not necessary to trigger contraction (McCleskey, 1985). This has led to the hypothesis that DHP binding proteins may play a dual role as calcium channels and as voltage sensors involved in the release of Ca^{2+} from the sarcoplasmic reticulum (Rios & Brum, 1987).

In skeletal muscle, as in cardiac muscle, the concentrations of DHPs required to affect calcium channel function are much higher than the nanomolar concentrations required to saturate the high-affinity binding sites that are measured in isolated membrane preparations [see Janis et al. (1987) and Triggle and Rampe (1989)]. This discrepancy may be explained, at least in part, by the voltage dependence of DHP binding (see Discussion). Models in which DHPs stabilize different states of the calcium channel (Hess et al., 1984) do not, however, include the possible contribution from lower affinity DHP binding sites that have been identified in several membrane preparations [see Janis et al. (1987), Glossmann and Striessnig (1988), and Langs et al. (1989)]. In many cases, this low-affinity binding has been attributed not to calcium channels but to other membrane proteins such as adenosine transporters (Glossmann & Striessnig, 1988).

The best established mechanism for regulation of L-type calcium channels is by second-messenger-mediated phosphorylation/dephosphorylation-dependent events [reviewed by Hosey and Lazdunski (1988)]. More recently, it has been shown that several ion channels, including calcium channels, may be directly regulated by guanine nucleotide binding (G) proteins [reviewed by Brown and Birnbaumer (1988)]. The G protein that is stimulatory to adenylate cyclase, G_s, has been shown to directly stimulate DHP-sensitive calcium channels in skeletal muscle transverse tubules (Yatani et al., 1988) in a manner independent of phosphorylation events.

In the present study, the kinetics of dihydropyridine binding to membrane preparations from rabbit skeletal muscle have been investigated. Evidence is provided for the existence of both high- and low-affinity binding sites. Binding of DHP agonists and antagonists to the high-affinity sites is unaffected by the guanine nucleotide analogues GTPγS and GDPβS.

† This work was supported by USPHS Grant GM-42375. S.M.J.D. is a Scholar of the Alberta Heritage Foundation for Medical Research.
* Author to whom correspondence should be addressed.

[1] Abbreviations: DHP, 1,4-dihydropyridine; G protein, guanine nucleotide binding protein; G_i, GTP binding protein inhibitory to adenylate cyclase; G_s, GTP binding protein stimulatory to adenylate cyclase; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/91/0430-5716$02.50/0

However, GTPγS appears to stimulate the binding of agonists, but not of antagonists, to the low-affinity sites.

Materials and Methods

Materials. (+)-[^{3}H]PN200-110 (75–87 mCi/mmol) was from DuPont, New England Nuclear. (±)-Bay K8644 and (±)-nitrendipine were generously provided by Dr. A. Scriabine (Miles Laboratories, New Haven, CT). The isomers (+)-PN200-110 and (−)-PN200-110 were generously provided by Sandoz Ltd. (Basel, Switzerland). Nifedipine, GTPγS, GDPβS, and other nucleotides were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Microsomal Membranes. Microsomal membranes were prepared from the back and hind leg muscles of small (2–3 lb) New Zealand White rabbits as previously described (Dunn, 1989). Protein concentrations were measured by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA). Binding activities for (+)-[^{3}H]PN200-110 in these preparations were 6–8 pmol/mg of protein.

Binding of (+)-[^{3}H]PN200-110. The binding of (+)-[^{3}H]PN200-110 at equilibrium and in kinetic experiments was measured in filtration assays in which the samples were shielded from light to minimize ligand photolysis. Specific details are given in the text and figure legends, but briefly, microsomal membranes (0.002–0.05 mg/mL) were incubated with (+)-[^{3}H]PN200-110, and at the appropriate times, 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 addition of 5 mL of ACS (Amersham Canada Ltd., Oakville, Ontario) scintillation fluid, the filters were counted for ^{3}H. The buffer used in most experiments was 25 mM Hepes–Tris/1 mM $CaCl_2$, pH 7.4, but in experiments in which the effects of guanine nucleotides were investigated, the buffer was 25 mM Hepes–Tris/2 mM $MgCl_2$, pH 7.4. Unless otherwise stated, all experiments were carried out at room temperature (23 ± 2 °C).

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. When full time courses were measured, dissociation was initiated either by the addition of 1 μM (±)-nitrendipine and filtering of 0.5-mL aliquots at appropriate times as described above or by 20-fold dilution of the preequilibrated membranes into buffer (and drugs as indicated) and filtering of 10-mL aliquots at the appropriate times. Initial rate data were obtained by addition of 1 mL of the membranes, preequilibrated with (+)-[^{3}H]PN200-110, to 5 μL of ethanol containing the desired concentration of drug. Thus, all samples, including controls, contained ethanol at a final concentration of 0.5%. Aliquots of 0.2 mL were removed and filtered after 0.25, 1, 1.5, and 2 min. In experiments in which GTPγS or GDPβS were used, the nucleotides were incubated with microsomal membranes for 5 min at room temperature prior to the addition of (+)-[^{3}H]PN200-110. At the concentrations of unlabeled DHPs used (0.1–100 μM), the drugs remained soluble in aqueous medium as verified by the lack of increase in turbidity measured at 550 nm.

Data Analysis. Initial rate dissociation data were analyzed by linear regression of the first-order log plots of ln (B_t/B_0) versus time where B_t is the amount of (+)-[^{3}H]PN200-110 bound at time t after initiation of dissociation by dilution and/or addition of excess unlabeled ligand and B_0 is the amount bound prior to initiation of dissociation. Full time courses of association and dissociation were analyzed by nonlinear regression techniques adapted from Bevington (1969).

Results

Kinetics of (+)-[^{3}H]PN200-110 Binding to Skeletal Muscle Membranes. The kinetics of association of (+)-[^{3}H]PN200-110 binding have been investigated under pseudo-first-order conditions in which the concentration of muscle membranes was low (2–5 μg/mL) and the concentration of ligand (0.2–3.0 nM) exceeded the density of binding sites by at least 10-fold. At all ligand concentrations, the association reaction did not significantly deviate from a single-exponential model (data not shown). The measured rate constant increased linearly with ligand concentration, suggesting that association is a simple bimolecular reaction:

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL$$

Under pseudo-first-order conditions, the above model predicts that the rate constant, k_{app}, will vary with (+)-[^{3}H]PN200-110 concentration, L, according to

$$k_{app} = k_1[L] + k_{-1}$$

Values of k_1 and k_{-1} have been estimated to be 4.7×10^6 M^{-1} s^{-1} and 0.0037 s^{-1}, respectively. It should, however, be noted that dissociation rate constants measured in this manner are subject to large error. Dissociation rates have therefore also been measured directly by triggering dissociation by the addition of excess unlabeled nitrendipine (1 μM) to the preformed complex. The dissociation reaction appeared to be a monoexponential process, and the estimated dissociation rate constant of 0.00134 s^{-1} was lower than that estimated from the association kinetics. This could be interpreted in terms of a conformational change following the formation of the binary complex. Attempts have been made to identify such an isomerization by studying the kinetics of dissociation under preequilibrium conditions, i.e., prior to the formation of the equilibrium complex. However, the rate of dissociation was identical when the dissociation was initiated by addition of 1 μM nitrendipine after 1, 2, 5, and 40 min of incubation (data not shown). Thus, no evidence for a conformational transition has been obtained, and given the intrinsic errors associated with determination of dissociation rates from association kinetics, it is likely that the observed discrepancy may not be significant. When the value for k_{-1} obtained by competition is used, the overall dissociation constant (k_{-1}/k_1) may be estimated to be 0.28 nM, in excellent agreement with previous estimates of 0.30 ± 0.05 nM obtained in equilibrium experiments (Dunn, 1989). Thus, the kinetics of (+)-[^{3}H]-PN200-110 binding to skeletal muscle DHP receptors appear to conform to a simple bimolecular model. This contrasts with the more complicated mechanism of binding of DHPs to rat brain membranes in which the receptor appears to exist in two interconvertible states, only one of which binds DHPs with high affinity (Weiland & Oswald, 1985).

Acceleration of the Rate of Dissociation of (+)-[^{3}H]-PN200-110 by Micromolar Concentrations of Other DHPs. Although the kinetics of (+)-[^{3}H]PN200-110 binding to its high-affinity sites appear to conform to a simple bimolecular model, detailed analysis of the dissociation kinetics has revealed a number of interesting complexities. If indeed the binding mechanism is simple and involves a homogeneous population of binding sites, it would be expected that the measured rate of dissociation of (+)-[^{3}H]PN200-110 would be identical whether dissociation was induced by infinite dilution or by competition with unlabeled DHPs. The results illustrated in Figure 1 demonstrate that this is not the case. When the preformed complex between 1 nM (+)-[^{3}H]PN200-110 and 50 μg/mL membrane protein was diluted 20-fold into buffer

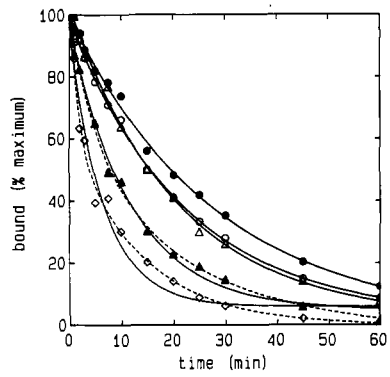

FIGURE 1: Effect of unlabeled (±)-nitrendipine on the dissociation of (+)-[^{3}H]PN200-110. 1 nM (+)-[^{3}H]PN200-110 was incubated with 50 μg/mL muscle membranes in 25 mM Hepes–Tris/1 mM $CaCl_2$, pH 7.4, for 40 min at room temperature prior to initiation of dissociation by 20-fold dilution into buffer alone (●) or buffer containing nitrendipine at a final concentration of 0.3 (○), 1.0 (△), 3 (▲), or 30 (◊) μM (±)-nitrendipine. At the time incubated, 10-mL aliquots were filtered as described under Materials and Methods. Solid lines are calculated from the best fits by a single exponential, and dissociation rates were 0.036 (●), 0.045 (○), 0.048 (△), 0.084 (▲), and 0.152 (◊) min^{-1}. The dashed lines show improved fits by a two-exponential model, and best-fit parameters for (▲) gave two rate constants for 0.2 and 0.042 min^{-1} with 43% of the dissociation occurring in the faster phase, and best-fit parameters for (◊) were 0.4 and 0.073 min^{-1} with 53% occurring in the faster phase.

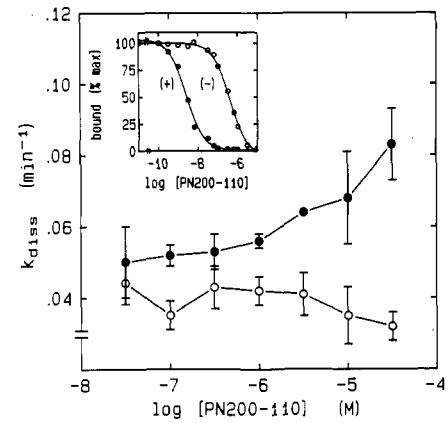

FIGURE 2: Effect of (+)-PN200-110 (●) and (−)-PN200-110 (○) on acceleration of (+)-[^{3}H]PN200-110 dissociation from its high-affinity sites. Data were obtained from experiments as described in the legend to Figure 1. Values for k_{diss} were obtained from fitting of the dissociation data to a single-exponential equation. As described in the text, this gives a qualitative measure of the ability of (+)-PN200-110 but not (−)-PN200-110 to accelerate the dissociation of the radiolabeled ligand. The inset shows effects of (+)- and (−)-PN200-110 on the high-affinity binding of (+)-[^{3}H]PN200-110. Membranes (50 μg/mL) were incubated with 1 nM (+)-[^{3}H]-PN200-110 and the indicated concentrations of unlabeled ligands for 60 min at room temperature, after which bound radiolabel was estimated by filtration assay. Data were fit by an equation assuming a single population of high-affinity binding sites: % bound = maximum bound/(1 + [I]/IC_{50}) where I is the concentration of displacing ligand. Best-fit values for IC_{50} were 2.5 nM and 0.4 μM for (+)- and (−)-PN200-110, respectively.

alone, the rate of dissociation was 0.03 ± 0.01 min^{-1} (n = 4). However, since these conditions are rather far from approximating infinite dilution, it is expected that the true dissociation rate constant is underestimated due to some rebinding of the radiolabeled ligand after dissociation. Dilution into buffer containing 30 nM (±)-nitrendipine, a concentration which, in the dilution mixture at equilibrium, would be expected to saturate >98% of the high-affinity binding sites, slightly accelerated the apparent dissociation rate to 0.042 ± 0.006 min^{-1}. This can readily be explained by prevention of rebinding of the radiolabeled ligand. Increasing the concentration of (±)-nitrendipine in the dilution mixture between 0.03 and 1 μM did not have much effect on the dissociation rate, and no deviations from monoexponential kinetics were observed (Figure 1). However, at concentrations of competing ligand greater than 1 μM, the rate of (+)-[^{3}H]PN200-110 dissociation was markedly increased, and the kinetics became perceptibly biphasic. These data suggest that the competing nitrendipine may bind to sites that are ostensibly of low affinity and must be allosterically linked to the high-affinity sites since their occupancy accelerates the rate of dissociation of (+)-[^{3}H]PN200-110. Under such circumstances, it would be expected that, under conditions of partial saturation of the low-affinity sites, the kinetics of dissociation of the radiolabeled ligand would be biphasic, the faster and slower components reflecting dissociation from receptors with low-affinity sites occupied or unoccupied, respectively. The data illustrated in Figure 1 are consistent with this notion with stimulated and unstimulated dissociation rate constants of approximately 0.3 and 0.05 min^{-1}. Quantitative analysis of the dissociation kinetics is, unfortunately, precluded by the closeness of the two rates which results in high correlation among the kinetic parameters, in addition to experimental difficulties associated with the poor time resolution of filtration assays and the limited aqueous solubility of DHPs. Qualitative observations, however, suggest that the accelerating effect of (±)-nitrendipine on the dissociation rate occurs over the concentration range of 1–100 μM, with a midpoint around 10 μM. In similar experiments, (±)-Bay K8644, a DHP agonist, and nifedipine, another DHP antagonist, have been found to similarly accelerate the rate of dissociation of (+)-[^{3}H]PN200-110 from its high-affinity sites, over similar concentration ranges (data not shown).

Stereospecificity of Dihydropyridine Binding. Identification of low (micromolar)-affinity binding sites is complicated by the possibility of nonspecific effects occurring at such high ligand concentrations. DHPs are lipophilic drugs and thus are likely to partition into the membranes. This could presumably result in nonspecific interactions with the DHP binding proteins. This possibility was investigated by measuring the binding properties of the two stereoisomers, (+)- and (−)-PN200-110, and the results described below favor the notion that the effects on dissociation rates observed at high DHP concentration arise from a specific rather than a nonspecific interaction.

High-affinity binding of DHPs is stereospecific, and the (+) enantiomer of PN200-110 is considerably more potent than the (−) enantiomer in displacing (+)-[^{3}H]PN200-110 in competition experiments (Figure 2, inset). These results are in agreement with previous studies using skeletal muscle (Glossmann et al., 1985) and cardiac muscle membranes (Maan & Hosey, 1987). The stereospecificity of the "low affinity" binding sites has been investigated by measuring the ability of high concentrations of the two enantiomers to accelerate the dissociation of 1 nM (+)-[^{3}H]PN200-110 from its high-affinity sites as described above for other DHPs. In these experiments, 20-fold dilution of the preformed membrane-radiolabeled ligand complex into buffer containing concentrations of (+)-PN200-110 greater than micromolar clearly increased the rate of dissociation. However, the (−) isomer had no effect on the dissociation rate (Figure 2). By measuring initial rates of dissociation as described below, it has been possible to extend the concentration range of (−)-PN200-110 tested to 200 μM, but this also was without effect. Thus, both high-affinity binding and the effects of the low-affinity binding component detected in dissociation experiments are stereospecific.

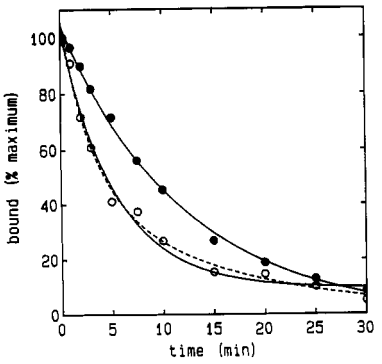

FIGURE 3: Effect of GTPγS on dissociation of (+)-[^{3}H]PN200-110 induced by dilution into buffer containing 1 μM (±)-Bay K8644. Muscle membranes (50 μg/mL) were preincubated with 1 nM (+)-[^{3}H]PN200-110 in the absence (●) or presence (○) of GTPγS for 40 min at room temperature in 25 mM Hepes–Tris, pH 7.4, and 2 mM $MgCl_2$. Dissociation was initiated by 20-fold dilution into buffer containing 1 μM (±)-Bay K8644. Solid lines are calculated from the best-fit parameters from fitting of a single-exponential equation as in Figure 2, giving rate constants of 0.081 and 0.185 min^{-1} in the absence and presence of GTPγS, respectively. The dashed line is calculated from a two-exponential fit with rate constants of 0.32 and 0.064 min^{-1} with the faster phase contributing 57% of the total amplitude. The experiment shown in representative of three such determinations.

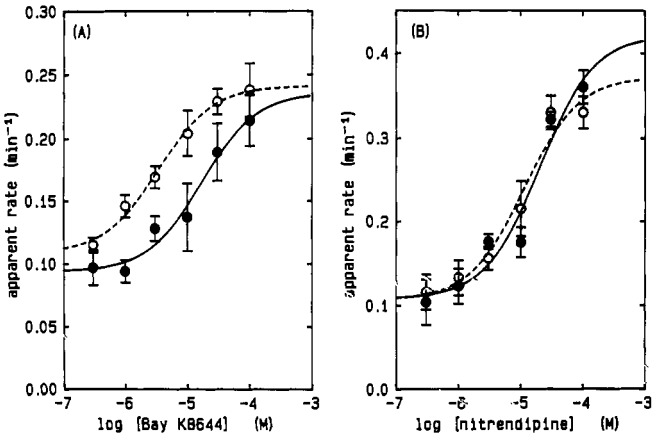

FIGURE 4: Effect of 10 μM GTPγS on the acceleration of (+)-[^{3}H]PN200-110 dissociation induced by (±)-Bay K8644 (A) and (±)-nitrendipine (B). Muscle membranes (50 μg/mL) in 25 mM Hepes–Tris/2 mM $MgCl_2$, pH 7.4, were incubated with 1 nM (+)-[^{3}H]PN200-110 in the absence (closed circles) or presence of 10 μM GTPγS for 40 min at room temperature prior to initiation of dissociation by rapid addition of 1 mL into 5 μL to give the final indicated concentration of competing ligand. The amount of radiolabeled ligand bound was measured during the first 2 min of dissociation as described in the text. Values of k_{diss} given were obtained from semilogarithmic plots assuming that dissociation follows first-order kinetics (see text). As described in the text, this is a simplifying assumption, and values of k_{diss} are not true dissociation rate constants. Data are pooled from five separate experiments, and each point represents the mean ± standard deviation of at least three determinations.

Effects of Guanine Nucleotides on DHP Binding. Recently there has been a great deal of interest in the role of G proteins that directly regulate ion channels independently of cellular second messengers [see Brown and Birnbaumer (1988)]. Since, in regulatory systems involving G proteins, the binding of GTP frequently alters the interaction of ligands with their receptors (Rodbell, 1980), we have investigated the effects of GTPγS and GDPβS, a pseudoirreversible activator and a competitive inhibitor of G proteins, respectively, on the properties of DHP binding to skeletal muscle membranes.

The amount of (+)-[^{3}H]PN200-110 bound when 1 nM was incubated with 50 μg/mL membrane protein was unaffected by the presence of GTPγS (0–30 μM) in the incubation medium. Equilibrium binding of unlabeled drugs to high-affinity DHP sites has been measured by their ability to compete with (+)-[^{3}H]PN200-110 binding. GTPγS, at a concentration of 10 μM, had no effect on the competition curves for either (±)-nitrendipine or (±)-Bay K8644, and 10 μM GDPβS was similarly found to be without effect on (±)-Bay K8644 competition. In other experiments, 10 μM GTPγS did not affect the ability of R4407 (a DHP antagonist) nor its isomer R5147 (an agonist) to compete for high-affinity sites. Thus, at these concentrations, no evidence has been obtained for an effect of guanine nucleotides on high-affinity DHP binding.

As described above, both (±)-Bay K8644 and (±)-nitrendipine, at greater than micromolar concentrations, increase the rate of dissociation of (+)-[^{3}H]PN200-110, and this is presumably a consequence of a protein conformational change induced by DHP occupancy of low-affinity sites. The effects of GTPγS and GDPβS on low-affinity binding have been investigated by measuring the ability of (±)-Bay K8644 and (±)-nitrendipine to accelerate dissociation. In preliminary experiments, 10 μM GTPγS was found to significantly increase the effectiveness of 1 μM (±)-Bay K8644 at accelerating dissociation of the radiolabeled ligand (Figure 3). This effect has been examined more closely by studying initial rates of dissociation. In these experiments, 1 nM (+)-[^{3}H]-PN200-110 was preincubated with 50 μg/mL muscle membranes in the presence or absence of 10 μM guanine nucleotide. After 40 min at room temperature, dissociation was initiated by rapidly mixing 1 mL of the incubation mixture with 5 μL of ethanol containing sufficient competing drug to give the desired final concentration. Dissociation was monitored over the next 2 min as described under Materials and Methods. From the first-order rates, dose–response curves for the acceleration of the dissociation rate induced by (±)-Bay K8644 and (±)-nitrendipine may be obtained (Figure 4). It should be noted that, although these data provide a reasonable description of the phenomenon, the rates given are not true rate constants. This is unavoidable since, as noted above, under conditions in which the low-affinity sites are not saturated, the dissociation kinetics are biphasic, whereas this analysis assumes monophasic dissociation. However, when a first-order approximation is used, the midpoint of the maximum effect ($E_{0.5}$) occurs at approximately 10–20 μM for both (±)-Bay K8644 and (±)-nitrendipine. These results are therefore in good agreement with the full time courses of dissociation such as those illustrated in Figure 1. In the presence of GTPγS, the curve for (±)-Bay K8644 is shifted to the left ($E_{0.5}$ approximately 3 μM) but that for (±)-nitrendipine is unaltered (Figure 4). GDPβS had no effect on the ability of either (±)-Bay K8644 or (±)-nitrendipine to accelerate the dissociation kinetics (data not shown).

Specificity of the Nucleotide Effect. In order to investigate whether the apparent low-affinity binding of (±)-Bay K8644 is sensitive specifically to GTPγS, the effects of several other nucleotides (GTP, GDP, ATP) on the ability of (±)-Bay K8644 to accelerate dissociation of previously bound [^{3}H]-PN200-110 have been studied. In these experiments, muscle membranes (50 μg/mL) were incubated with 1 nM [^{3}H]-PN200-110 in the presence of 10 μM nucleotide for 40 min prior to initiation of dissociation by rapid dilution into 1 μM (±)-Bay K8644 and measurement of the amount of residual bound radiolabeled ligand over the first 3 min of dissociation, i.e., conditions similar to those shown in Figure 4. In the absence of added nucleotide, the apparent first-order dissociation rate was 0.12 ± 0.02 min^{-1} (n = 9). Neither GDP

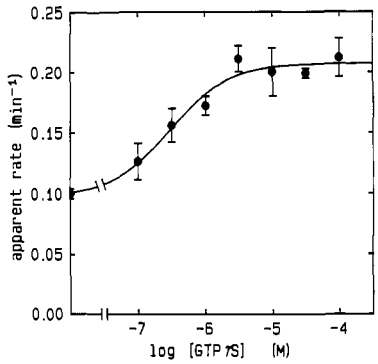

FIGURE 5: Effect of GTPγS concentration on the acceleration of dissociation of (+)-[^{3}H]PN200-110 induced by 10 μM (±)-Bay K8644. Data were obtained from initial rates of dissociation as described in the legend to Figure 4. Muscle membranes (50 μg/mL) in 25 mM Hepes–Tris/2 mM $MgCl_2$, pH 7.4, were incubated with 1 nM (+)-[^{3}H]PN200-110 in the presence of the indicated concentration of GTPγS prior to initiation of dissociation by addition of 10 μM (±)-Bay K8644. Data are average values from two separate experiments in which each determination was made in duplicate.

(0.12 ± 0.01 min^{-1}, n = 6) nor ATP (0.12 ± 0.02 min^{-1}, n = 7) in the preincubation mixture had any significant effect, similar to the lack of effect in the presence of GDPβS (0.11 ± 0.01 min^{-1}, n = 8). Both GTP and GTPγS did, however, accelerate the dissociation rate, giving dissociation rates of 0.15 ± 0.02 (n = 6) and 0.19 ± 0.02 (n = 9) min^{-1}, respectively. Thus, the effects on low-affinity binding of Bay K8644 seem to be specific for GTP and its analogues.

Effect of GTPγS on the Acceleration of (+)-[^{3}H]*PN200-110 Dissociation Induced by* (±)-*Bay K8644.* In order to provide a description of the concentrations of GTPγS required to stimulate the low-affinity effects of (±)-Bay K8644, the effect of varying the GTPγS concentration in the preincubation on the initial rate of dissociation stimulated by 10 μM (±)-Bay K8644 has been examined (Figure 5). Although again the rates are not true rate constants, it is clear that the midpoint of the dose–response curves for the stimulatory effect occurs at about 0.3 μM.

Discussion

In many previous studies of the properties of voltage-dependent calcium channels, attempts have been made to correlate the binding of DHPs with their effects on calcium channel function. However, only in smooth muscle has an essentially 1:1 correlation been demonstrated between binding and effects on depolarization-induced mechanical responses (Bolger et al., 1983). In skeletal and cardiac muscle, there are serious quantitative discrepancies, and drugs have been shown to display high-affinity binding and low-affinity pharmacology [see Hosey and Lazdunski (1988) and Triggle and Rampe (1989)]. To some extent, this discrepancy may be explained by the voltage dependence of DHP binding. In electrophysiological studies using cardiac preparations, Sanguinetti and Kass (1984) and Bean (1984) found that the ability of several drugs to inhibit calcium currents was increased under depolarized conditions, leading to the suggestion that DHPs bind more tightly to an inactivated state favored by depolarization. Increased binding of DHPs in the depolarized state has also been observed in direct binding studies using cardiac muscle cells, cardiac sarcolemma preparations, and intact skeletal muscle [reviewed by Hosey and Lazdunski (1988)].

The presence of multiple binding sites having different affinities may also explain the discrepancy between binding site affinity and functionally effective concentrations of ligands. The data presented here demonstrate the presence of multiple binding sites for 1,4-dihydropyridines in the DHP binding protein from rabbit skeletal muscle. In addition to the high-affinity (nanomolar K_d) binding sites that are readily measured in equilibrium binding studies, there appear to be additional low-affinity sites, which under preequilibrium conditions have K_d values for several DHPs in the range of 10 μM. Occupancy of the latter sites accelerates dissociation of (+)-[^{3}H]-PN200-110 bound to the high-affinity sites, suggesting that the two classes of sites are conformationally coupled and are associated with the same protein complex.

In a previous study, low-affinity binding sites for DHPs were identified and correlated with calcium channel function (Brown et al., 1986). In guinea pig myocytes, the effects of nitrendipine and Bay K8644 were biphasic and membrane potential dependent, and the results were interpreted in terms of the presence of two types of DHP sites related to calcium channels, one with low affinity (K_d approximately 1 μM) and one with high affinity (K_d approximately 1 nM). On the basis of their findings, Brown et al. (1986) suggested that the low-affinity sites mediate stimulatory effects due to prolonged channel openings and that the high-affinity sites may be either stimulatory or inhibitory depending on membrane potential.

The observation of multiple binding sites of different affinities for calcium channel ligands in skeletal muscle membranes is not unique to the DHPs. In addition to binding sites for DHPs, L-type calcium channels carry distinct binding sites for structurally unrelated channel blockers, including the phenylalkylamines, such as verapamil, and the benzothiazepines, such as diltiazem (Striessnig et al., 1986). In a previous study, the rate of dissociation of [^{3}H]verapamil was shown to be increased by dilution into buffer containing micromolar concentrations of unlabeled verapamil isomers and by diltiazem, but not by nitrendipine (Galizzi et al., 1984). The presence of both high- and low-affinity binding sites has also been demonstrated in other systems, including β-adrenergic (Limbird et al., 1975), insulin (DeMeyts et al., 1976), and nicotinic acetylcholine receptors (Dunn & Raftery, 1982). Thus, regulation of channel activity by means of ligand binding to multiple sites with different affinities and functions may be a common mechanism and may provide a level of control that is not attainable through a single binding site.

Recently, evidence has been obtained for G-protein regulation of calcium channels by a direct mechanism that does not involve changes in intracellular second messengers [reviewed by Brown and Birnbaumer (1988)]. A stimulatory, direct control of cardiac (Yatani et al., 1987) and skeletal (Yatani et al., 1988) muscle calcium channels by G_s has been proposed. Transverse tubule membranes have been shown to contain endogenous G proteins of both the G_s and the G_i type (Scherer et al., 1987; Toutant et al., 1988, 1990). By studying the properties of transverse tubule membranes incorporated in planar bilayers, Yatani et al. (1988) have shown that activation of an endogenous G protein by GTPγS results in the direct activation of calcium channels. GTPγS, at a concentration of 100 μM, stimulated calcium channels by a factor of 10–20 in the absence of Bay K8644 and by 2–3 in the presence of 3 μM Bay K8644. Guanine nucleotides have also been shown to change the binding of DHPs to their receptors in cardiac muscle (Triggle et al., 1986), and the nonhydrolyzable GTP analogue GMP-PNP was found to stimulate the binding of Bay K8644, but not nitrendipine, to rat cortical membranes (Bergamashi et al., 1988) and to PC12 cell membranes (Bergamashi et al., 1990). These findings are in agreement with the electrophysiological experiments of Scott

and Dolphin (1988), who showed that GTPγS potentiated the agonist effects of Bay K8644 on calcium currents in rat dorsal root ganglion cells.

In the present study, no evidence has been obtained for the ability of guanine nucleotides to modulate the binding of DHPs to their high-affinity binding sites in skeletal muscle. This observation is in agreement with a previous report (Galizzi et al., 1984) in which GTP, at a concentration of 0.13 mM, did not affect the binding of [^{3}H]nitrendipine to skeletal muscle transverse tubule membranes, although several guanyl nucleotides inhibited the binding of [^{3}H]verapamil to its high-affinity sites. In contrast to the lack of effect on high-affinity binding, GTPγS and GTP appear to modulate the binding of Bay K8644, but not nitrendipine, to the low-affinity sites on the skeletal muscle receptor. The concentrations of GTPγS that affected Bay K8644 binding ($K_{0.5}$ approximately 0.3 μM) are lower than those found to activate calcium channels in planar bilayers (Yatani et al., 1988), although they are similar to those shown to stimulate Bay K8644 binding to rat brain membranes (Bergamashi et al., 1988). Further studies of the mechanism underlying the coupling between GTPγS binding and effects on DHP binding will be required to explain this quantitative discrepancy.

In conclusion, measurement of DHP binding kinetics has revealed the presence of both high- and low-affinity binding sites in membrane preparations from rabbit skeletal muscle. There is some interaction between the two classes of sites, since occupancy of the sites of lower affinity accelerates the dissociation of radiolabeled (+)-PN200-110 from the high-affinity sites. GTPγS, a GTP analogue that activates G proteins, modulates the binding of the agonist, Bay K8644, but not the antagonist, nitrendipine, to the low-affinity sites. These observations suggest a role for a G protein in the regulation of DHP binding to low-affinity sites that are likely to be associated with voltage-dependent calcium channels.

References

Bean, B. P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6388–6392.

Bean, B. P. (1989) *Annu. Rev. Physiol. 51*, 367–384.

Bergamashi, S., Govoni, S., Cominetti, P., Parenti, M., & Trabucchi, M. (1988) *Biochem. Biophys. Res. Commun. 156*, 1279–1286.

Bergamashi, S., Trabucchi, M., Battaini, F., Parenti, M., Schettini, G., Meucci, O., & Govoni, S. (1990) *Eur. Neurol. 30*, 16–20.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Bolger, G. T., Gengo, P., Klockowski, R., Luchowski, E., Siegel, H., Janis, R. A., Triggle, A. M., & Triggle, D. J. (1983) *J. Pharmacol. Exp. Ther. 225*, 291–309.

Brown, A. M., & Birnbaumer, L. (1988) *Am. J. Physiol. 254*, H401–H410.

Brown, A. M., Kunze, D. L., & Yatani, A. (1986) *J. Physiol. 379*, 495–514.

Curtis, B. M., & Catterall, W. A. (1986) *Biochemistry 25*, 2077–2083.

DeMeyts, P., Bianco, A. R., & Roth, J. (1976) *J. Biol. Chem. 251*, 1877–1888.

Dunn, S. M. J. (1989) *J. Biol. Chem. 264*, 11053–11060.

Dunn, S. M. J., & Raftery, M. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6757–6761.

Flockerzi, V., Oeken, H.-J., Hofmann, F., Pelzer, D., Cavalie, A., & Trautwein, W. (1986) *Nature 323*, 66–68.

Fosset, M., Jaimovich, E., Delpont, E., & Lazdunski, M. (1983) *J. Biol. Chem. 258*, 6086–6092.

Galizzi, J.-P., Fosset, M., & Lazdunski, M. (1984) *Eur. J. Biochem. 144*, 211–215.

Glossmann, H., & Striessnig, J. (1988) *Vitam. Horm. 44*, 155–328.

Glossmann, H., Linn, T., Rombusch, M., & Ferry, D. R. (1983) *FEBS Lett. 160*, 226–232.

Glossmann, H., Ferry, D. R., Goll, A., Striessnig, J., & Zernig, G. (1985) *Arzneim-Forsch. 35*, 1917–1935.

Hess, P. (1990) *Annu. Rev. Neurosci. 13*, 337–356.

Hess, P., Lansman, J. B., & Tsien, R. W. (1984) *Nature 311*, 538–544.

Hosey, M. M., & Lazdunski, M. (1988) *J. Membr. Biol. 104*, 81–105.

Janis, R. A., Silver, P., & Triggle, D. J. (1987) *Adv. Drug Res. 16*, 301–591.

Langs, D. A., Janis, R. A., & Triggle, D. J. (1989) *Med. Res. Rev. 9*, 123–180.

Limbird, L. E., DeMeyts, P., & Lefkowitz, R. J. (1975) *Biochem. Biophys. Res. Commun. 64*, 1160–1168.

Maan, A. C., & Hosey, M. M. (1987) *Circ. Res. 61*, 379–388.

McCleskey, E. W. (1985) *J. Physiol. (London) 361*, 231–249.

Rios, E., & Brum, G. (1987) *Nature 325*, 717–720.

Rodbell, M. (1980) *Nature 284*, 17–22.

Sanguinetti, M. C., & Kass, R. S. (1984) *Circ. Res. 55*, 336–348.

Scherer, N. M., Toro, M.-J., Entman, M. L., & Birnbaumer, L. (1987) *Arch. Biochem. Biophys. 259*, 431–440.

Scott, R. H., & Dolphin, A. C. (1988) *Neurosci. Lett. 89*, 170–175.

Smith, J. S., McKenna, E. J., Ma, J., Vilven, J., Vaghy, P. L., Schwartz, A., & Coronado, R. (1987) *Biochemistry 26*, 7182–7188.

Striessnig, J., Goll, A., Moosburger, K., & Glossmann, H. (1986) *FEBS Lett. 197*, 204–210.

Toutant, M., Barhanin, J., Bockaert, J., & Rouot, B. (1988) *Biochem. J. 254*, 405–409.

Toutant, M., Gabrion, J., Vandaele, S., Peraldi-Roux, S., Barhanin, J., Bockaert, J., & Rouot, B. (1990) *EMBO J. 9*, 363–369.

Triggle, D. J., & Rampe, D. (1989) *Trends Pharmacol. Sci. 10*, 507–511.

Triggle, D. J., Skattebol, A., Rampe, D., Joclyn, A., & Gengo, P. (1986) in *New Insights Into Cell and Membrane Transport Processes* (Poste, G., & Crooke, S. T., Eds.) pp 125, Plenum Press, New York.

Weiland, G. A., & Oswald, R. E. (1985) *J. Biol. Chem. 260*, 8456–8464.

Yatani, A., Codina, J., Reeves, J. P., Birnbaumer, L., & Brown, A. M. (1987) *Science 238*, 1288–1292.

Yatani, A., Imoto, Y., Codina, J., Hamilton, S. L., Brown, A. M., & Birnbaumer, L. (1988) *J. Biol. Chem. 263*, 9887–9895.