# L-Type Calcium Channels: Binding Domains for Dihydropyridines and Benzothiazepines Are Located in Close Proximity to Each Other†

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*Received June 7, 1996; Revised Manuscript Received January 21, 1997*⊗

ABSTRACT: We investigated the binding of a fluorescent diltiazem analogue (3*R*,4*S*)-*cis*-1-[2-[[3-[[3-[4,4-difluoro-3a,4-dihydro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacen-3-yl]propionyl]amino]propyl]amino]ethyl]-1,3,4,5-tetrahydro-3-hydroxy-4-(4-methoxyphenyl)-6-(trifluoromethyl)-2*H*-1-benzazepin-2-one (DMBODIPY-BAZ) to L-type $Ca^{2+}$ channels in the presence of different 1,4-dihydropyridines (DHPs) by using fluorescence resonance energy transfer (FRET) [Brauns, T., Cai, Z.-W., Kimball, S. D., Kang, H.-C., Haugland, R. P., Berger, W., Berjukov, S., Hering, S., Glossmann, H., & Striessnig, J. (1995) *Biochemistry 34*, 3461]. When channels are occupied with DMBODIPY-BAZ, a rapid fluorescence change occurred upon addition of different DHPs. The direction of this intensity modulation was found to be only dependent on the chemical composition of the dihydropyridine employed. DHPs containing a nitro group decreased, whereas others (e.g., isradipine) enhanced the fluorescence signal. In addition, all DHPs markedly decreased the association rate constant for DMBODIPY-BAZ without affecting equilibrium binding. Both observations together are best explained by a steric model where the DHP binding site is located in close proximity to the accession pathway of DMBODIPY-BAZ.

Voltage-gated $Ca^{2+}$ channels control the depolarization-induced influx of extracellular $Ca^{2+}$ into electrically excitable cells. At least six different channel types (L, N, T, P, Q, R) have been described (Tsien et al., 1991). They can be distinguished by their biophysical properties as well as their sensitivity to organic $Ca^{2+}$ antagonists and peptide toxins. These differences are explained by the existence of multiple classes of $\alpha_1$ subunits ($\alpha_{1A}$−$\alpha_{1E}$, $\alpha_{1S}$) forming the voltage-sensitive ion conducting pore of these channel types (Birnbaumer et al., 1994).

L-type $Ca^{2+}$ channels are sensitive to various chemical classes of organic channel blockers, termed $Ca^{2+}$ antagonists. DHPs[1] (e.g., isradipine), PAAs (e.g., verapamil) and BTZs (e.g., diltiazem) are clinically used to treat cardiovascular disorders. Moreover, labeled and unlabeled derivatives of these drugs represent important tools to study the structure and function of these channels.

An important prerequisite for the understanding of the molecular mechanism underlying the modulation of channel function by $Ca^{2+}$ antagonists is the knowledge about the organization of their drug binding domains on the $\alpha_1$ subunit. Radioligand binding studies predicted the existence of distinct interaction domains for DHPs, PAAs, and BTZs that are coupled to each other via noncompetitive mechanisms (Glossmann & Striessnig, 1990). Electrophysiological studies using membrane-impermeable drugs further refined the localization of these binding domains with respect to the membrane bilayer. The DHP and BTZ binding domain were found to be located on the extracellular side of $\alpha_1$, whereas PAAs interact with a site only accessible from the cytoplasmic surface of the channel (Hescheler et al., 1982). Antibody mapping studies with photoaffinity-labeled channels revealed that amino acid residues responsible for high-affinity DHP and PAA binding are located within pore-forming regions in repeats III and IV of $\alpha_1$ (Striessnig et al., 1990; Nakayama et al., 1991; Striessnig et al., 1991). These findings were recently confirmed and extended using chimeric (Grabner et al., 1996; Döring et al., 1996) or singly mutated $\alpha_1$ subunits (Hockerman et al., 1995).

Despite the growing structural information about the regions within the primary structure involved in the formation of the drug binding domains, several important questions remain unresolved. First, how far apart are drug binding domains located on the $\alpha_1$ subunit, especially if they are known to reside on the same side of the channel (DHPs, BTZs)? Second, what is the molecular basis for the

† This work was supported by grants from the Fonds zur Förderung der Wissenschaftlichen Forschung (S6601 to H.G., S6602 to J.S.) and the Dr. Legerlotz Foundation (to J.S.).

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1997.

[1] Abbreviations: AFU, arbitrary fluorescence units; BTZ, benzothiazepine; Bz-BAZ, (3*R*,4*S*)-*cis*-1-[2-[[3-(benzoylamino)propyl]amino]ethyl]-1,3,4,5-tetrahydro-3-hydroxy-4-(4-methoxyphenyl)-6-(trifluoromethyl)-2*H*-1-benzazepin-2-one; DHP, 1,4-dihydropyridine; DMBODIPY-BAZ, (3*R*,4*S*)-*cis*-1-[2-[[3-[[3-[4,4-difluoro-3a,4-dihydro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacen-3-yl]propionyl]amino]propyl]amino]ethyl]-1,3,4,5-tetrahydro-3-hydroxy-4-(4-methoxyphenyl)-6-(trifluoromethyl)-2*H*-1-benzazepin-2-one; DMSO, dimethylsulfoxide; FRET, fluorescence resonance energy transfer; $EC_{50}$, concentration causing half-maximal effect; $k_1$, $k_{app}$, bimolecular association rate constant and apparent association rate constant; $k_{-1}$, dissociation rate constant; PAA, phenylalkylamine.

S0006-2960(96)01358-X CCC: $14.00 

noncompetitive interaction between the different binding domains?

Here we exploit the fluorescent properties of a novel diltiazem analogue, DMBODIPY-BAZ (Brauns et al., 1995), to address these questions. By quantitatively analyzing the effects of various DHPs on the fluorescent properties as well as on the binding kinetics of $\alpha_1$-bound DMBODIPY-BAZ we provide strong evidence for a steric interaction between DHPs and BTZs. This suggests that the binding domains of these drugs are localized in close proximity to each other on the extracellular side of the channel.

## MATERIALS AND METHODS

### *Chemicals*

Unlabeled calcium antagonists were kindly provided by Dr. Traut (Knoll AG, Ludwigshafen, Germany) and Sandoz AG (Basel, Switzerland); DMBODIPY-BAZ and Bz-BAZ were synthesized as described before (Brauns et al., 1995); digitonin (special grade) was from Biosynth AG (Staad, Switzerland); bovine serum albumin (essentially fatty acid free) and other chemicals were from Sigma (Deisenhofen, Germany).

### *Calcium Channel Preparations*

Partially purified L-type calcium channels from rabbit skeletal muscle were prepared by affinity chromatography of digitonin-solubilized membranes on Wheat-Germ-Lectin Sepharose according to standard protocols (Brauns et al., 1995).

### *Fluorescent DMBODIPY-BAZ Binding Assays*

*FRET-Binding Assay.* Binding of DMBODIPY-BAZ (20 nM) was determined in buffer A (50 mM Tris/HCl, pH 7.4, 0.1% (w/v) digitonin, 0.25 mg/mL bovine serum albumin) as described employing $Ca^{2+}$ channel protein concentrations of 0.02−0.035 mg/mL (80 pmol/mg of (+)-isradipine binding sites (Berger et al., 1994; Brauns et al., 1995), yielding receptor concentrations of 1.6−2.8 nM). The FRET signal was measured simultaneously in up to four temperature-controlled (25 °C) and magnetically stirred quartz cuvettes using an excitation wavelength of 285 nm (slit width = 4 nm) and recording emission at 517 nm (slit width 8 nm) in a Perkin-Elmer LS50B spectrofluorimeter. Data acquisition rates were 0.7 data points/min.

*Measurement of DHP Effects on DMBODIPY-BAZ Fluorescence by Direct Excitation at 488 nm.* The fluorescence signal after excitation at 488 nm does not discriminate between bound and free ligand. As DHPs only affect the fluorescence of bound ligand, experiments were carried out under conditions where a high ratio of bound to free ligand was achieved. These were obtained by employing high receptor (9.6 nM) and low ligand concentrations (5−8.3 nM). After binding equilibrium was achieved, DHPs were added to a final concentration of 3 $\mu$M and changes in fluorescence were recorded at 517 nm (0.7 data points/min). The means of 10 data points before and after addition of DHP were calculated to quantify increases or decreases in fluorescence. No DHP-induced DMBODIPY-BAZ fluorescence changes were observed in the absence of channel protein ($n$ = 3).

*Stopped-Flow Binding Measurements.* Highly time-resolved measurements of DHP-induced changes in FRET-intensity were recorded in a Hi-Tech SF-61MX stopped-flow apparatus. $Ca^{2+}$ channel protein (4 nM) was preincubated (1.5 h, 25 °C) with DMBODIPY-BAZ (20 nM) in buffer A. The mixture (65 $\mu$L) was then mixed with an equal volume of buffer A (control) or a drug solution prepared in buffer A. The FRET intensity was measured (excitation = 285 nm, emission cut-off filter = 495 nm) at a data acquisition rate of typically >20 points/s. Photobleaching was negligible during acquisition time.

### *Quantitative Analysis of Receptor−Ligand Kinetics*

All kinetic experiments were analyzed on the basis of their differential equations employing the program FACSIMILE (Prinz & Striessnig, 1993). The strategy for obtaining the global fit shown in Figure 3 was the following: In a first step, four association reactions were recorded after the addition of 10, 30, 100, and 300 nM DMBODIPY-BAZ to 1.6 nM (determined as DHP-binding sites) of $Ca^{2+}$ channels. These experiments (data not shown) showed a fluorescence increase, followed by a slow decrease. This slow inactivation of binding activity had been observed before with other calcium channel ligands and was explained by the slow denaturation of the channel protein in detergent solution (Striessnig et al., 1986; Schneider et al., 1991). It could not be attributed to photobleaching of the fluorochrome as shown in control experiments.

These binding data were simultaneously fitted to an association reaction accounting for the irreversible binding inactivation according to Scheme 1. R indicates the $Ca^{2+}$ channel, L the fluorescent ligand, and RX inactivated channels. A global fit was achieved with reaction 1 for all ligand concentrations, but not with the same rate constants, $k_x$, for the channel inactivation, which varied between experiments (observed range 3.9 − 26 × $10^{-6}$ $s^{-1}$).

Scheme 1

$$R + L \underset{}{\overset{1}{\rightleftarrows}} LR; \quad R \xrightarrow{x} RX; \quad LR \xrightarrow{x} RX + L$$

Scheme 2

$$\begin{array}{lll} R \overset{1}{\rightleftarrows} LR & & R \xrightarrow{x} RX \\ \quad\updownarrow 2 \qquad \updownarrow 3 & & LR \xrightarrow{x} RX + L \\ RD \underset{}{\overset{4}{\rightleftarrows}} LRD & & RD \xrightarrow{x} RX + D \end{array}$$

Having thus determined the rate constants for reaction 1 in Scheme 1, we analyzed the kinetic experiments in the presence of the DHP isradipine (shown in Figure 3) on the basis of Scheme 2: D indicates the nonfluorescent DHP ((+)- or (−)-isradipine), RD the DHP-channel complex, and LRD the ternary complex. The addition of ligands is not explicitly shown for each reaction step. A ternary complex LRD is required in order to account for the observed FRET enhancement (see below, Figures 1 and 2). The following assumptions were made for the fitting procedure: Rate constants for the decay of R, LR, and RD were assumed to be identical. No decay of the ternary complex LRD was required. For reactions 2 and 3 only their relative affinities and not their rate constants were determined from the experiments shown in Figure 3. The association rate constant for (+)-isradipine was taken from previous experiments [for $k_2$ see Knaus et al. (1992a) Striessnig et al. (1986)]. $k_3$ was determined in

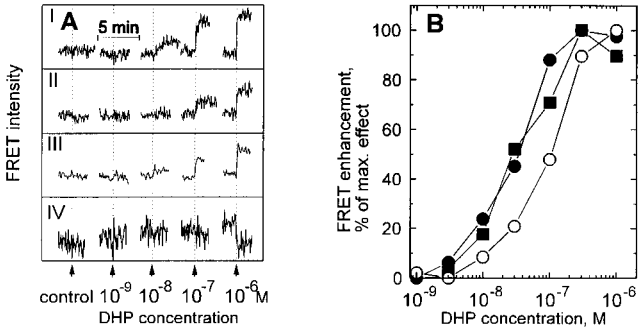


FIGURE 1: Modulation of FRET intensity by different DHPs. (**A**) Partially purified $Ca^{2+}$ channel protein (1.6−2.8 nM) was incubated with 20 nM DMBODIPY-BAZ in buffer A until equilibrium was reached (1.5 h, 25 °C). One milliliter aliquots were transferred to quartz cuvettes and the FRET signal was recorded (excitation at 285 nm, emission at 517 nm). DHPs dissolved in 5 $\mu$L of DMSO or DMSO alone (control) were added to the indicated final concentrations. I, (+)-isradipine; II, (−)-isradipine; III, darodipine; IV, (+)-202-791. Arrow indicates time of DHP addition. (**B**) Concentration-dependence of FRET enhancement by (+)-isradipine (●), (−)-isradipine (○) and darodipine (■). The amplitude of the fluorescence increase was determined as in panel A and normalized with respect to the maximal effect.

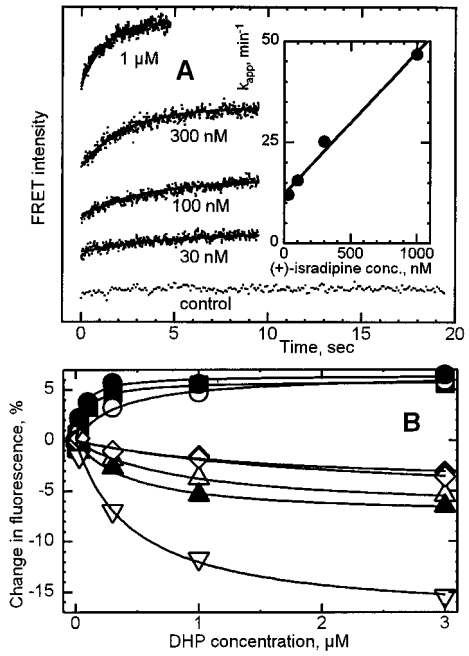


FIGURE 2: Stopped-flow measurements of the FRET modulation (**A**) Partially purified $Ca^{2+}$ channel protein (4 nM) was incubated with 20 nM DMBODIPY-BAZ in buffer A (25 °C, 1.5 h) and rapidly mixed with increasing concentrations of (+)-isradipine (30 nM to 1 $\mu$M) in a stopped-flow apparatus. FRET was measured every 10−100 ms. The points represent means of six to eight measurements. A monophasic association function was fitted to the data (solid line) to yield the following apparent association rates, $k_{app}$: 30 nM, 0.2 $s^{-1}$; 100 nM, 0.26 $s^{-1}$; 300 nM, 0.42 $s^{-1}$; 1 $\mu$M, 0.78 $s^{-1}$. (Inset) Linear regression of a plot of $k_{app}$ vs (+)-isradipine concentration yielded a $k_1$ of $5.8 \times 10^5$ $M^{-1}$ $s^{-1}$ and a $k_{-1}$ of 0.21 $s^{-1}$. (**B**) Modulation of FRET by several DHPs as observed in stopped-flow experiments. The relative change in total fluorescence intensity after 25 s is plotted against the applied DHP concentration for (●) (+)- and (○) (−)-isradipine, (■) darodipine, (◆) (+)- and (◇) (−)-202-791, (▲) (+)- and (△) (−)-nitrendipine, and (▽) (−)-niguldipine.

stopped-flow experiments (see below). Constants for (−)-isradipine could not be determined and were assumed to be $10^7$ $M^{-1}$ $s^{-1}$ in accordance with a diffusion controlled association. $k_2$ and $k_3$ were held constant during the fitting

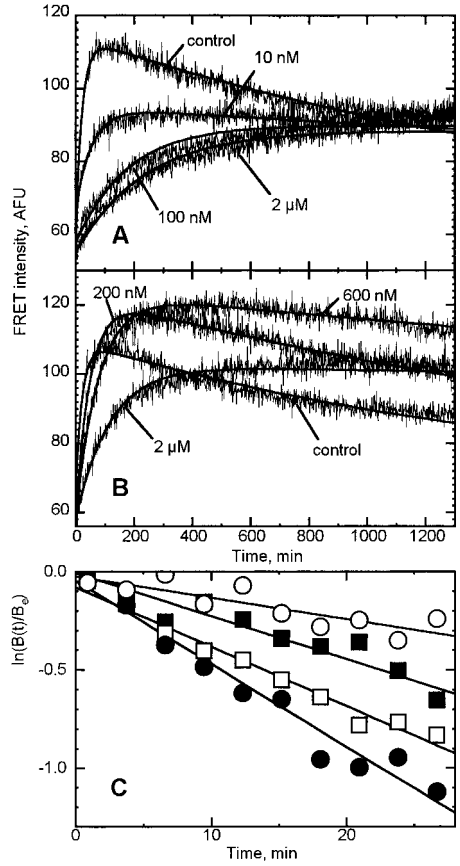


FIGURE 3: Effect of isradipine enantiomers on association and dissociation kinetics of DMBODIPY-BAZ as measured by FRET (excitation, 285 nm; emission, 517 nm). (**A, B**) Partially purified $Ca^{2+}$ channel protein (1.6 nM final concentration) was rapidly mixed with buffer A containing 20 nM DMBODIPY-BAZ and the indicated concentrations of (+)-isradipine (panel A) or (−)-isradipine (panel B). Heavy lines represent association curves calculated from the kinetic parameters derived by least-squares fitting to reaction scheme 2 (see Materials and Methods section). The following $k_x$ values were used ($s^{-1}$): (+)-isradipine, control, $8.5 \times 10^{-6}$, 10 nM, $2.7 \times 10^{-6}$, 100 nM, $2.3 \times 10^{-7}$, 2 $\mu$M, $2.0 \times 10^{-7}$; (−)-isradipine, control, $1.0 \times 10^{-5}$, 200 nM, $8.2 \times 10^{-6}$, 600 nM $3.3 \times 10^{-6}$, 2 $\mu$M $9.7 \times 10^{-7}$. Note, that irreversible inactivation also decreases with isradipine concentration indicating possible protection by the DHP. (**C**) After equilibrium was reached, an excess of 1 $\mu$M unlabeled Bz-BAZ was added in order to measure DMBODIPY-BAZ dissociation. The semilogarithmic plot of the FRET signal reveals a decrease in dissociation rate with increasing concentrations (as indicated) of (+)-isradipine present. $k_{-1}$ Values were calculated as the slope of the regression line: (●) control, $7.0 \times 10^{-4}$ $s^{-1}$; (□) 10 nM, $5.0 \times 10^{-4}$ $s^{-1}$; (■) 100 nM, $3.7 \times 10^{-4}$ $s^{-1}$; (○) 2 $\mu$M, $1.8 \times 10^{-4}$ $s^{-1}$. One of three similar experiments is shown.

procedure. The fluorescence yield of the ternary complex LRD had been determined experimentally from the independent analysis of our FRET-enhancement experiments and was kept constant. It was 1.2 times the fluorescence of the binary complex LR (Figure 1). As a starting parameter the association reaction 4 was assumed to be 10 times slower than the association reaction 1. Its affinity ($K_{d4}$) was initially assumed to be equal to $K_{d1}$. With these initial parameters all rate constants shown in Table 1 were derived from a global fit. The last part of the experiments in the presence of (+)-isradipine (Figure 3A) could not be accounted for with this simple reaction scheme. Instead of extending the scheme, we excluded the data points obtained after 1000 min from the global fitting procedure.

Table 1: Rate Constants Determined from a Global Fit of Reaction Scheme 2 to the Experimental Data Shown in Figure 3

| | (+)-isradipine | (−)-isradipine |
|---|---|---|
| $K_{d1}$ (nM) | 25.6 | 25.6 |
| $k_1$ ($M^{-1}$ $s^{-1}$) | $1.6 \times 10^4$ | $1.6 \times 10^4$ |
| $k_{-1}$ ($s^{-1}$) | $4.2 \times 10^{-4}$ | $4.2 \times 10^{-4}$ |
| $K_{d2}$ (nM) | 1.77 | 109.5 |
| $k_2$ ($M^{-1}$ $s^{-1}$)[a] | $4.5 \times 10^5$ | $10^7$ |
| $k_{-2}$ | ND | ND |
| $K_{d3}$ (nM) | 5.8 | 109.5 |
| $k_3$ ($M^{-1}$ $s^{-1}$)[a] | $5.8 \times 10^5$ | $10^7$ |
| $k_{-3}$ | ND | ND |
| $K_{d4}$ (nM) | 84 | 25.6 |
| $k_4$ ($M^{-1}$ $s^{-1}$) | $6.5 \times 10^2$ | $2.4 \times 10^3$ |
| $k_{-4}$ ($s^{-1}$) | $5.5 \times 10^{-5}$ | $6.1 \times 10^{-5}$ |

[a] Held constant. [b] ND, not determined (see Materials and Methods section).

*Statistics*

Data are given as range or means ±SD for the indicated number of experiments.

## RESULTS AND DISCUSSION

We have recently described the development of a novel binding assay using the fluorescent diltiazem analogue DMBODIPY-BAZ (Brauns et al., 1995) as a ligand specific for the BTZ binding site. This assay is based on FRET (Stryer & Haugland, 1967) between one or more tryptophan residues of the L-type $Ca^{2+}$ channel $\alpha_1$ subunit and the DMBODIPY fluorophore of DMBODIPY-BAZ. It allows the direct quantification of specifically bound ligand with high time resolution and under equilibrium conditions. Binding occurred with high affinity ($K_d$ = 25 nM) and was reversible (Brauns et al., 1995). We have now studied the interaction between two classes of channel ligands (BTZs and DHPs) that are able to bind to their domains on the $\alpha_1$ subunit simultaneously.

*DHPs Modulate DMBODIPY-BAZ FRET Fluorescence*

DMBODIPY-BAZ was added to partially purified skeletal muscle $Ca^{2+}$ channels, and its association reaction was directly followed as a FRET fluorescence increase (Brauns et al., 1995). After equilibrium was achieved, we added the benzoxadiazol-DHP (+)-isradipine to the preformed DMBODIPY-BAZ−channel complex. Nanomolar concentrations of (+)-isradipine increased the FRET signal in a concentration-dependent manner (Figure 1A). A similar increase was also observed for the respective (−)-enantiomer and the achiral isradipine analogue darodipine. The increase was not due to a stimulation of ligand binding as the fluorescence change occurred in the time course of seconds, much faster than the observed association rate required for the formation of new DMBODIPY-BAZ−receptor complexes ((Brauns et al., 1995), for $k_{+1}$ see below). The DHP-induced increase in FRET fluorescence was not accompanied by a detectable shift in the maximum of the emission spectrum (not shown). Addition of other DHPs such as (+)-202-791 resulted in a decrease of the FRET emission (see Figure 1A, trace IV).

The isradipine effect critically depended on the presence of DMBODIPY-BAZ−channel complexes. It was absent when channel protein was omitted from the incubation mixture ($n$ = 10) or when DHP was added after complete dissociation of the specific binding component with a nonfluorescent competitor (Bz-BAZ, (Brauns et al., 1995)) ($n$ = 5). When added at different times during the association time course, the amplitude of the DHP-induced FRET increase was proportional to the concentration of bound DMBODIPY-BAZ ($n$ = 2). Moreover, the effect was absent when, instead of the BTZ site, the PAA binding domain of the channel was labeled with the fluorescent verapamil analogue DMBODIPY-PAA (Knaus et al., 1992b) ($n$ = 2, data not shown).

Taken together, these experiments show that the FRET enhancement occurs only when the DHP binds to the DMBODIPY-BAZ-occupied channel to form a ternary complex.

This implies that the amplitude of the FRET increase is saturable and that its rate (reflecting the association of the DHP to the DMBODIPY-BAZ-channel complex) must depend on the concentration of added DHP. Figure 1B illustrates the saturability of the DHP-induced FRET enhancement. A small but significant ($p > 0.05$) stereoselectivity was observed for stimulation by the two isradipine enantiomers ((+)-isradipine: $EC_{50}$ = 29.0 ± 11.2 nM, $n$ = 6; (−)-isradipine: $EC_{50}$ = 106 ± 14.6 nM, $n$ = 5).

The time course of the FRET increase in Figure 1A suggests a very high association rate of the DHP. Stopped-flow experiments clearly resolved this formation of the ternary complex at a higher time resolution. Figure 2A demonstrates that the apparent association rate ($k_{app}$) increased with the concentration of (+)-isradipine added. From a plot of $k_{app}$ vs isradipine concentration we calculated a $k_1$ of 6.7 ± 0.93 × $10^5$ $M^{-1}$ $s^{-1}$ and a $k_{-1}$ of 0.17 ± 0.04 $s^{-1}$ ($n$ = 3), yielding a kinetically derived $K_d$ of 254 nM. This $K_d$ is about 2 orders of magnitude higher than reported for high-affinity DHP binding and in agreement with the low apparent affinity of (+)-isradipine for FRET enhancement as determined in equilibrium experiments (Figure 1). Taken together, our results indicate that isradipine forms an initial, weakly stereoselective contact with the channel protein that mediates the enhancement of FRET fluorescence.

To determine if the DHP-induced change in FRET fluorescence depends on DHP structure, we examined in stopped-flow experiments the effect of a number of DHPs on FRET fluorescence (Figure 2B). The DHPs differed with respect to their substituents in positions 3 or 5 of the DHP ring and on the aromatic system linked to position 4 (for structural formulae see Figure 4). FRET increase was only found for the isradipine enantiomers and darodipine. In contrast, the enantiomers of 202-791 both caused a similar concentration-dependent decrease of FRET fluorescence. Note, that in functional experiments (−)-202-791 acts as a channel blocker, whereas the (+)-enantiomer is an activator. A FRET-reducing effect of similar magnitude was also observed for both enantiomers of the antagonist/agonist pair of (+)- and (−)-BayK 8644 (17−20% and 12−14% reduction, respectively, $n$ = 2). The channel blockers (−)-nitrendipine and (+)-niguldipine were also inhibitory. Like for (+)-isradipine half-maximal effects were observed in a concentration range about 100-fold higher than their reported $K_d$ (Berger et al., 1994; Knaus et al., 1992b; Vaghy et al.,

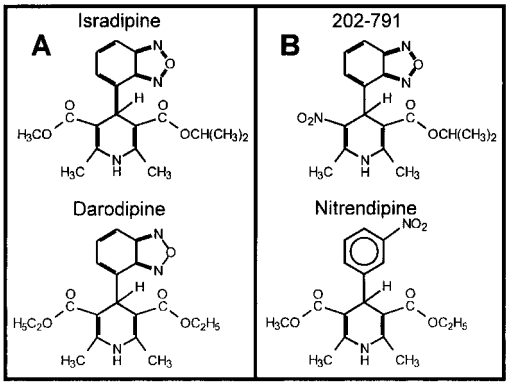


FIGURE 4: Structural formulae of ligands binding specifically to the DHP binding site: (**A**) Drugs found to enhance FRET from channel protein to DMBODIPY-BAZ are the (+)- and (−)-enantiomer of isradipine and the achiral darodipine. (**B**) Structurally similar DHPs inducing FRET decrease are both enantiomers of 202-791 and nitrendipine.

1987; Striessnig et al., 1985) for channel binding (Figure 2B). All DHPs that decrease the FRET signal contained a nitro group on the phenyl ring or in position C3 or C5.

The observed changes in FRET fluorescence could also be explained if the various DHP inhibitors bind to a site very close to a tryptophan that is the major excited-state energy donor to the bound DMBODIPY-BAZ. It is therefore conceivable that the DHPs could modulate the quantum yield of this tryptophan, thereby modulating the magnitude of the FRET without changing interfluorophore distance. This could all occur at a distance from the DMBODIPY-BAZ site. To exclude this possibility we investigated the effect of DHPs under conditions where fluorescence changes of bound DMBODIPY-BAZ were measured by direct excitation at 488 nm (see Methods). For all four benzoxadiazol DHPs tested ((+)-isradipine, darodipine, (+)-202-791, (−)-202-791), the fluorescence change was qualitatively the same as observed for FRET analysis ($n \geq 2$ for each DHP tested; absolute changes ranging from 1 to 6% of total fluorescence). Consequently, the FRET-induced change cannot (or not only) be explained by DHP effects on tryptophan quantum yield. DHP effects on DMBODIPY-BAZ were experimentally more difficult to determine (see Methods) by excitation at 488 nm. Therefore, no detailed kinetic analysis was performed under these experimental conditions.

### *Steric Interaction Model for DHP and DMBODIPY-BAZ Binding*

A possible explanation for our experimental findings could be an allosteric mechanism linking the two drug binding sites. This would require DHP-induced conformational changes of the channel molecule and thus changing the efficiency of FRET between DMBODIPY-BAZ and one or more tryptophan residues of the $\alpha_1$ subunit. Although such a mechanism cannot be ruled out completely, our experimental evidence argues against such a model: First, DHP agonists and antagonists represent optical antipodes able to stabilize different channel conformations that correspond to open and closed conformations in functional experiments (Glossmann & Striessnig, 1990). However, FRET fluorescence changes did not correlate with the opposite pharmacological activity of these stereoisomers as the FRET decrease was seen by both, agonistic and antagonistic enantiomers of 202-791 and BayK 8644. Second, among DHP antagonists both enhancement and inhibition of FRET fluorescence was observed. An allosteric model predicts that DHPs stabilize (or induce) a channel conformation that displays a different energy transfer efficiency from the channel tryptophans to the DMBODIPY-BAZ fluorophore. Our finding of opposite effects on FRET fluorescence by different DHPs would therefore require that different DHPs stabilize different channel conformations. This possibility is very unlikely because opposite effects on FRET fluorescence were observed for structurally highly related antagonists (e.g. (+)-isradipine and (−)-202-791). This minor difference in chemical structure is not expected to induce long-distance allosteric effects distinguishable as FRET fluorescence changes. Third, if DHP-induced changes of channel conformation would exist they should also affect FRET to bound fluorescent calcium antagonists of other chemical classes. As mentioned above, such DHP-induced changes were not detectable after labeling of the channel with a DMBODIPY-coupled verapamil analogue, DMBODIPY-PAA. Therefore, overall changes in channel conformation are very unlikely. Fourth, qualitatively similar effects on the fluorescence of bound ligand were observed when DMBODIPY-BAZ was directly excited at 488 nm, not involving FRET. These experiments rule out that DHP effects on FRET fluorescence are only due to changes in tryptophan quantum yield.

In contrast, our data are most easily explained by assuming that the change of DMBODIPY-BAZ FRET fluorescence results from close interaction with DHP molecules. This explains why all tested DHPs carrying a nitro group (Figure 4), a known fluorescence quencher (Lakowicz, 1983), lead to a decrease in the FRET signal. It implies that the binding domains for DHPs and DMBODIPY-BAZ must be located in close proximity to each other. Our data therefore strongly support a steric model for the interaction of these two classes of $Ca^{2+}$ channel ligands.

### *DHPs Slow the Binding Kinetics of DMBODIPY-BAZ*

The close proximity of the two drug molecules in the ternary complex could also explain the noncompetitive binding mechanisms observed earlier for the modulation of diltiazem binding by DHPs. In particular, DHPs are known to slow the dissociation of ligands from the benzothiazepine binding domain (Glossmann & Striessnig, 1990; Brauns et al., 1995). We therefore investigated the effects of the isradipine enantiomers on DMBODIPY-BAZ binding kinetics in more detail. The association time course for the formation of the DMBODIPY-BAZ−channel complex at 25 °C is illustrated in Figure 3. As shown previously, the association reaction followed second-order kinetics, the initial association rate increasing linearly with the concentration of ligand (Brauns et al., 1995). As discussed in the Materials and Methods section, the association reaction was accompanied by an irreversible inactivation of binding activity due to slow denaturation of the channel in detergent solution (Schneider et al., 1991).

The association rate of DMBODIPY-BAZ decreased in the presence of increasing concentrations of the unlabeled DHPs (+)- or (−)-isradipine (Figure 3). In the case of the more potent (Striessnig et al., 1986) (+)-isradipine, this effect was saturable at micromolar concentrations so that a similar decrease was observed for 0.1 and 2 $\mu$M (Figure 3A). In contrast to the slowing of the association rate, the maximal

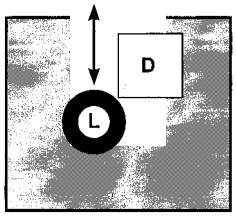


FIGURE 5: Steric model of DHP and BTZ binding. L indicates DMBODIPY-BAZ and D isradipine bound to the $\alpha_1$ subunit. Access to the L-binding site is hampered in the presence of D. The close proximity of the two ligands explains the drug−drug interaction that results in a modulation (enhancement or reduction) of the FRET emission.

binding was not decreased after prolonged incubation in the presence of (+)-isradipine. This was also confirmed in separate charcoal-binding assays (not shown). Note, that within the experimental time range no DHP-induced fluorescence increase (due to the higher fluorescence yield of the ternary complex) above control was observed in the presence of (+)-isradipine. This can be explained by the pronounced slowing of the DMBODIPY-BAZ association time course that would allow apparent stimulation only after an even longer incubation time. However, as the slowing of DMBODIPY-BAZ association was less pronounced for (−)-isradipine, the fluorescence increase became apparent.

Both enantiomers also decreased the dissociation rate of the DMBODIPY-BAZ−channel complex, when induced by the addition of a nonfluorescent competitor [Figure 3C, see also Brauns et al. (1995)]. Attempts to determine the effect of DHPs on the dissociation reaction of DMBODIPY-BAZ initiated by rapid dilution were not feasible due to the low specific binding signal obtained after dilution of preformed complexes in the cuvette.

Similar kinetic effects were induced by other DHP $Ca^{2+}$ channel ligands. All of them caused a concentration-dependent decrease of DMBODIPY-BAZ association (1 $\mu$M darodipine: 3−4.5-fold; (−)-nitrendipine, 4−8-fold; (+)-niguldipine, 4−5-fold; (−)-202-791, 4−6-fold; (+)-202-791, 2−3-fold; range, $n \geq 3$) and dissociation (data not shown) as described above for (+)-isradipine.

Our observations of a DHP-induced decrease of apparent association rates without a decrease of equilibrium binding (neither $K_d$ nor $B_{max}$) require a model that assumes simultaneous binding of BTZs and DHPs to distinct sites on the channel. The simplest reaction diagram of this kind is depicted in Scheme 2 (see Materials and Methods section). The experiments of Figure 3 were analyzed with this model and with one consistent set of parameters (global fit). The resulting rate constants are shown in Table 1. The values obtained for isradipine-free reaction 1 are in good accordance with the recently derived values for $K_{d1}$, $k_1$ and $k_{-1}$ (25 nM, $1.1 \pm 0.15 \times 10^4$ $M^{-1}$ $s^{-1}$, $9.2 \pm 2 \times 10^{-4}$ $s^{-1}$, respectively, from Brauns et al., (1995). As expected from Figure 3, the DMBODIPY-BAZ-association rate constant in the presence of isradipine ($k_4$) was markedly smaller than in its absence ($k_1$): $k_1/k_4 = 25$ and 6.7 in the case of (+)- and (−)-isradipine, respectively. This indicates that especially (+)-isradipine prevents rapid access of DMBODIPY-BAZ to its binding site, which can be explained easily by the model illustrated in Figure 5: If the binding site for DHPs (D) is localized within the access pathway of DMBODIPY-BAZ (L), then D can sterically hinder the access of L, explaining the observed decrease in its association rate. The close proximity of both ligands explains the fluorescence changes shown in Figures 1 and 2.

Another important finding of our study was that the FRET increase by (+)-isradipine is observed at drug concentrations 1−2 orders of magnitude above its reported $K_d$ value for partially purified skeletal muscle $Ca^{2+}$ channels. This implies that FRET enhancement occurs upon formation of a low affinity DHP - channel complex. In contrast to the high affinity binding of (+)-isradipine, the formation of this complex is only weakly (about 3-fold, Figure 1) stereoselective. Therefore, this low-affinity interaction must represent an initial binding step of isradipine that may be followed by further rearrangements of the drug−receptor complex leading to the high-affinity binding interaction. The discrepancy between the apparent affinity for FRET enhancement and reported $K_d$ (or $K_i$) values was mainly seen for (+)-isradipine (29 nM in equilibrium experiments in Figure 1 vs a $K_d$ of 1−2 nM) but was absent for (−)-isradipine (106 nM vs a $K_i$ of 120 nM, (Berger et al., 1994)). This indicates that after the formation of the "initial" complex only the (+)-enantiomer can form additional contacts that mediate stereoselective high affinity binding. These findings further support a multistep binding model previously proposed for the binding of a fluorescence-labeled DHP (Berger et al., 1994).

The above steric model is in accordance with the finding that DHPs and BTZs both approach their site from the extracellular side of the channel (Hering et al., 1993). If DHP binding occurs at a site in close proximity to the BTZ binding domain, the DHP molecule could interfere with the binding process of the bulky DMBODIPY-BAZ molecule. DMBODIPY-BAZ association and dissociation from the DHP-occupied channel would be blocked (or delayed). Such an "ordered binding mechanism" has recently been described for [$^3$H]*N*-methylscopolamine and vecuronium binding to the muscarinic acetylcholine receptor (Proska & Tucek, 1994). In another study the association and dissociation rate of *N*-methylacridinium binding to acetylcholinesterase was found to be decreased by up to 3 orders of magnitude in the presence of the snake venom fasciculin 2 (Rosenberry et al., 1996).

This model is also in accordance with our recent biochemical data (Kraus et al., 1996), which show that a photoreactive DMBODIPY-BAZ analogue photoincorporates into a region of skeletal muscle L-type calcium channel $\alpha_1$ subunits (IIIS6) which also forms part of the DHP binding domain. These results provide biochemical support for the close proximity of the DHP and BTZ binding domains and also suggest that the bulky side chains of these diltiazem derivatives are responsible for the drug−drug interaction described here.

Our results also explain findings on purified heart sarcolemmal membranes (Garcia et al., 1986) where PAA and diltiazem (as BTZ-specific ligand) binding were inhibited by treatment of the channel with dithiothreitol. Prebinding of the DHP nitrendipine to the channel prior to addition of dithiothreitol partially protected the BTZ binding site, but not the one for the PAA [$^3$H]verapamil. This differential influence of DHP binding on two distinct binding sites can easily be explained by a shielding effect of the DHP molecule burying the BTZ binding site in analogy to our above model.

*Implication for Channel Structure*

Our steric model gives rise to a new working hypothesis providing a basis for the future structural characterization of $Ca^{2+}$ channel drug binding domains. It predicts that structurally diverse L-type $Ca^{2+}$ antagonists (DHPs, PAAs, and BTZs) possess binding affinity to a restricted area ("hot-spot for drug *binding*") close to the extracellular, intracellular, or transmembrane regions of the channel pore. Recent work refining the localization of the DHP and PAA binding domain on the $\alpha_1$ subunit further supports this hypothesis. It was shown that both (noncompetitively coupled, (Glossmann & Striessnig, 1990)) binding domains are formed by stretches of primary structure located in close proximity within the pore-forming regions (Grabner et al., 1996; Hockerman et al., 1995) according to current folding models of $\alpha_1$ (Durell & Guy, 1996). This region of $\alpha_1$ undergoes defined voltage-induced changes upon channel opening and closing (Ellinor et al., 1993) and carries the channel's high-affinity $Ca^{2+}$ binding sites ($Ca^{2+}$ selectivity filter, (Yang et al., 1993)). It may therefore represent the channel's most efficient target for drug action ("hot-spot for drug *action*") where $Ca^{2+}$ antagonists could affect channel gating and/or calcium coordination (Mitterdorfer et al., 1995).

Further structural studies determining the localization of several other $Ca^{2+}$ antagonist drug binding domains on L-type channels will be necessary to confirm or refute our hypothesis. A detailed model of these domains will facilitate the development of nonpeptidergic, high-affinity blockers for other classes of voltage-gated $Ca^{2+}$ channels that could be valuable therapeutic agents for the treatment of chronic pain and cerebral ischemia (Miljanich & Ramachandran, 1995).

## ACKNOWLEDGMENT

We thank D. Reimer and E. Penz for providing purified calcium channel preparations and Dr. H.-C. Kang for DMBODIPY-BAZ.

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BI9613584