Benzothiazepinone Binding Domain of Purified L-Type Calcium Channels: Direct Labeling Using a Novel Fluorescent Diltiazem Analogue†

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ABSTRACT: We have synthesized a series of *N*-propylamino-substituted benzazepinones (NPSBs) as specific probes for the benzothiazepinone (BTZ) binding domain of muscle L-type calcium channels (LTCCs). NPSBs were identified which possess high affinity for the channel after purification. We synthesized a fluorescent NPSB, DMBODIPY-BAZ, as the first benz(othi)azepinone derivative known to reversibly label partially purified LTCCs. DMBODIPY-BAZ binds to the partially purified channel with high affinity (K_d = 25 nM, B_{max} = 580 pmol/mg of protein). Fluorescence resonance energy transfer (FRET) occurred between tryptophan residues of the channel protein and the DMBODIPY fluorophore upon specific drug binding. FRET was exploited to allow highly time-resolved detection of specific drug binding kinetics. We found that the dissociation half-life ($t_{1/2}$) of DMBODIPY-BAZ decreased with the concentration of an unlabeled competitor, which indicates ligand-induced accelerated dissociation. In contrast, $t_{1/2}$ was concentration-dependently increased by the dihydropyridine (DHP) (+)-isradipine. These kinetic properties of DMBODIPY-BAZ indicate that a high-affinity BTZ binding domain also exists on purified LTCCs. NPSBs represent novel tools to provide further insight into the molecular pharmacology of the BTZ binding domain on LTCCs.

Voltage-gated L-type calcium channels (LTCCs)[1] mediate depolarization-induced calcium entry into excitable cells. The resulting increase in intracellular free calcium controls important physiological processes, e.g., muscle contraction, cardiac excitability, and secretion. Organic compounds that block LTCCs are termed calcium antagonists; different chemical classes can be distinguished among them. Dihydropyridines (e.g., isradipine), phenylalkylamines (e.g., verapamil), and benzothiazepinones (e.g., diltiazem) are widely used in clinical practice to treat cardiovascular disorders (Kaczorowski et al., 1994).

Calcium antagonists block LTCCs after association with high-affinity binding domains on the pore-forming α_1-subunit of the heterooligomeric channel complex [for a review, see Kaczorowski et al. (1994)]. Reversible binding studies with radiolabeled calcium antagonists and membrane-bound calcium channels from cardiac (Garcia et al., 1986; King et al., 1988) or skeletal muscle T-tubule membranes (Striessnig & Glossmann, 1991) demonstrated the existence of separate binding domains for all three main classes of calcium antagonists. The purification of LTCCs from skeletal muscle has enabled studies to characterize these domains at the structural level. Photoaffinity probes (Striessnig et al., 1991) have allowed the direct irreversible labeling of regions for dihydropyridine (DHP) and phenylalkylamine (PAA) binding within the α_1-subunit. These two classes of drugs bind close to the extra- and intracellular opening of the channel pore, respectively. In contrast, no suitable probes exist for the reversible labeling of the benzothiazepinone (BTZ) binding domain on purified LTCCs. This is mainly due to a dramatic decrease in binding affinity for (+)-*cis*-diltiazem and its photoreactive derivatives (Naito et al., 1989; Striessnig et al., 1990; Glossmann et al., 1989) upon channel purification. Although photolabeling studies suggested that BTZs also interact with a site on the α_1-subunit (Naito et al., 1989; Striessnig et al., 1990), more detailed biochemical studies of this binding domain were so far impossible.

Here, we report the development of a new series of BTZ analogues (*N*-propylamino-substituted benzazepinones, NPSBs) that are potent blockers of LTCCs in intact muscle cells. In contrast to (+)-*cis*-diltiazem, their affinity increases upon channel purification. As these drugs tolerate bulky hydrophobic substituents without major loss in affinity, we were

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[1] Abbreviations: AFU, arbitrary fluorescence units; B_{max}, maximum density of binding sites; BSA, bovine serum albumin; BTZ, benzothiazepinone; 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; CBZ, carbobenzyloxy; 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, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; IC_{50}, concentration causing 50% binding inhibition; I_{Sr}, inward strontium current; k_{+1} and k_{app}, association rate constant and apparent association rate constant; k_{-1}, dissociation rate constant; LTCC, L-type calcium channel; NPSB, *N*-propylamino-substituted benzazepinone; PAA, phenylalkylamine; $t_{1/2}$, half-life time; BOC, butoxycarbonyl.

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able to synthesize the first known fluorescent diltiazem analogue. Using this ligand, a novel binding assay based on fluorescence resonance energy transfer (FRET) was developed. As the existence of a high-affinity benzothiazepinone binding domain on partially purified LTCCs can be unequivocally demonstrated, NPSBs will be valuable tools to study the molecular architecture of the BTZ binding domain in greater detail.

MATERIALS AND METHODS

Chemicals. Unlabeled calcium antagonists were kindly provided by Dr. Traut (Knoll AG, Ludwigshafen, Germany) and Sandoz AG (Basel, Switzerland); Tinuvin 770 was from Ciba-Geigy (Basel, Switzerland); digitonin (special grade) was from Biosynth AG (Staad, Switzerland); bovine serum albumin (BSA, essentially fatty acid free) and other chemicals were from Sigma Chemical Co. (Deisenhofen, Germany).

Synthesis of Bz-BAZ and DMBODIPY-BAZ (Figure 1). Compound **1a** (Floyd et al., 1992) (8.1 g, 23 mmol) was treated with NaH in dimethylformamide (DMF) (0 °C), followed by allyl bromide and chromatography on silica gel (EtOAc/hexanes) to give **1b** (8.4 g, 93%). Olefin **1b** (8.0 g) was treated with ozone (−78 °C) and dimethylsulfide, followed by silica gel chromatography to provide aldehyde **1c** (6.3 g, 78%).

Aldehyde **1c** (1.97 g, 5.0 mmol) was stirred with mono-CBZ 1,3-diaminopropane hydrochloride and $NaCNBH_3$ in MeOH to give the ω-CBZ-*N*-propylamino-substituted benzazepinone **1d** (1.87 g, 64%), which was reacted with di-*tert*-butyl dicarbonate to introduce the *tert*-BOC protecting group (1.64 g, 2.8 mmol). This *N*-*tert*-BOC, *N'*-CBZ derivative **2a** was hydrogenated over Pd/C (20%) to give the free amine of **2b** (3*R*,4*S*)-*cis*-1,3,4,5-tetrahydro-6-trifluoromethyl-3-hydroxy-1-[[2-[3-aminopropyl]]-2-[1,1-dimethylethoxycarbonyl]amino]ethyl-4-(4-methoxy)phenyl-2*H*-1-benzazepin-2-one (1.28 g, 87%). The *N*-benzoyl *N*-propylamino-substituted benzazepinone 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] was prepared by reacting **2b** (432 mg) with benzoyl cyanide (415 mg, 81%), and the *tert*-BOC protecting group was removed (150 mg) with HBr/acetic acid to give Bz-BAZ (144 mg, 98%).

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]was synthesized by coupling of **2b** (5 mg) with 4,4-difluoro-3a,4-dihydro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacen-3-ylpropionic acid, succinimidyl ester (4 mg), followed by removing the *tert*-BOC protecting group with trifluoroacetic acid at 0 °C. The pure DMBODIPY-BAZ was obtained as an orange-red solid (4 mg, 61%) after silica gel chromatography (10% methanol in chloroform).

Calcium Channel Preparations. Membranes enriched in skeletal muscle transverse-tubule membranes and partially purified calcium channels were prepared according to previously published procedures (Striessnig & Glossmann, 1991). The integrity of post-translationally processed (170 kDa) α_1-subunits in these preparations was verified by Coomassie staining of sodium dodecyl sulfate polyacrylamide gels and immunoblot analysis (Grabner et al., 1991).

Radioligand and Fluorescent DMBODIPY-BAZ Binding Assays. Radioligand binding assays to particulate or partially purified channels were carried out using previously published procedures (Striessnig & Glossmann, 1991). Binding of fluorescent DMBODIPY-BAZ was determined using a charcoal assay (Knaus et al., 1992b). DMBODIPY-BAZ and channel protein were incubated at 25 °C in incubation buffer (50 mM Tris/HCl, pH 7.4, 0.1% (w/v) digitonin, 0.25 mg/mL BSA) in a final volume of 1 mL in the absence and presence of unlabeled drugs. DMBODIPY-BAZ and other nonfluorescent drugs were directly added from serial dilutions made in dimethyl sulfoxide (DMSO). Final DMSO concentrations were kept below 1% (v/v), a concentration that did not affect ligand binding. To remove bound from free ligand, the incubation mixtures were cooled on melting ice for 3 min and then rapidly mixed with 0.25 mL of an ice-cold charcoal suspension (40 mg/mL in deionized water). After 10 min, the charcoal was removed by centrifugation at 15800 *g* for 5 min (5 °C). A total 0.075 mL of 1.5% (w/v) digitonin was added to the supernatant, and the fluorescence was measured in a Perkin-Elmer LS50B spectrofluorimeter in quartz cuvettes. The DMBODIPY-BAZ concentration before (total ligand concentration) and after charcoal separation (bound ligand) was determined from calibration curves relating arbitrary fluorescence units (AFU) to ligand concentration. Fluorescence was measured at 517 nm (slit width 2.5−10 nm dependent on ligand concentration) after excitation at 488 nm (slit width 4 nm). Nonspecific binding was determined in the presence of 1 μM Bz-BAZ and was subtracted from total binding to yield specific binding.

Determination of Channel-Bound DMBODIPY-BAZ Using FRET. Incubation conditions for equilibrium experiments were identical to those described above for the charcoal assay. The FRET signal was measured in quartz cuvettes at an emission wavelength of 517 nm (slit width 8 nm). In contrast to the charcoal assay, excitation was carried out at 285 nm (slit width 4 nm). This wavelength causes preferential excitation of tryptophan residues and direct excitation of the DMBODIPY-BAZ is almost absent (Figure 3A). The spectral overlap between the protein (i.e., tryptophan) emission spectrum and the DMBODIPY-BAZ excitation spectrum should theoretically allow FRET from tryptophans (energy donor) to the DMBODIPY group of the ligand (energy acceptor) provided the fluorophores are within the critical distance (5 nm) known to be required for FRET to occur (Stryer & Haugland, 1967). This criterion is more likely to be fulfilled for the bound than for the free ligand. As shown in the results section, the fluorescence signal obtained under these experimental conditions was indeed preferentially obtained from channel-bound drug. On-line kinetic measurements using FRET were carried out in magnetically stirred, temperature-controlled (25 °C) quartz cuvettes at time resolutions of 2−60 measurements per minute. Association reactions were started by the addition of partially purified Ca^{2+} channel preparation to DMBODIPY-BAZ in incubation buffer. Dissociation kinetics were induced by the addition of 5 μL of Bz-BAZ in DMSO to 1 mL of the incubation mixture. In control experiments, no photobleaching of DMBODIPY-BAZ or protein fluorescence were detectable after 4 h of irradiation at 285 or 488 nm under the above experimental conditions.

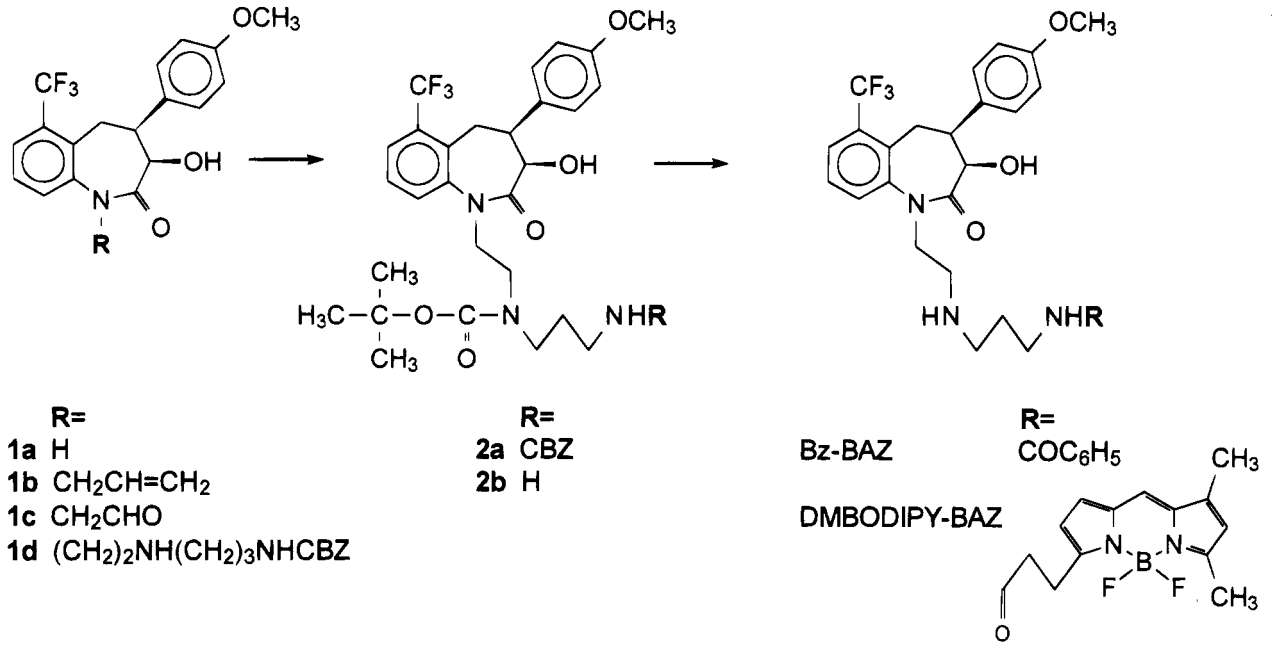

FIGURE 1: Synthetic pathway for NPSBs.

Whole Cell Patch–Clamp Experiments. A7r5 cells were cultured as described (Hering et al., 1993). Inward Sr^{2+} currents (I_{Sr}) through L-type Ca^{2+} channels were recorded at 22–25 °C using the whole cell configuration of the patch–clamp technique (Hamill et al., 1981). Patch pipets with a resistance of 1–4 MΩ were made from borosilicate glass and filled with pipet solution. The pipet solution contained (in mM) the following: CsCl 60, CsOH 60, aspartate 60, $MgCl_2$ 2, HEPES 10, and EGTA 10 adjusted to pH 7.25 with CsOH. I_{Sr} were measured in high external Sr^{2+} solution containing (in mM) the following: $SrCl_2$ 10, *N*-methyl-D-glucamine 170, HEPES 10, glucose 20, 4-aminopyridine 4, tetraethylammonium chloride 27, and $MgCl_2$ 3, buffered to pH 7.3 with methanesulfonic acid. Data were filtered at 2 kHz (four-pole Bessel filter). Leak currents were subtracted digitally (using average values of scaled leakage currents elicited by a 10-mV hyperpolarizing pulse) or electronically by means of an analogue circuit. Exchange of the bath solution (i.e., external drug application) was achieved within 10 s.

Data Analysis. Binding inhibition curves were analyzed by nonlinear least-square fitting of the experimental data to the general dose–response equation (DeLean et al., 1978) to yield IC_{50} values and slope factors. Association and dissociation data were fitted to equations describing exponential association and dissociation reactions. For further details see text and figure legends. K_i values were calculated according to Cheng and Prussoff (1973).

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

RESULTS

Synthesis of Novel Diltiazem Analogues with High Affinity for Partially Purified Calcium Channels. The decrease of binding affinity upon purification of Ca^{2+} channels for (+)-*cis*-[^{3}H]diltiazem (Naito et al., 1989; Striessnig et al., 1990; Glossmann et al., 1989) prompted us to develop a labeled analogue with high affinity for purified LTCCs. As a first step, the binding properties of a series of novel benzazepinones for membrane-bound and purified LTCCs were analyzed. Benzazepinones are structurally closely related to diltiazem-like BTZs, possess calcium antagonist activity, and bind to the BTZ binding domain of LTCCs (Kimball et al., 1992; Hering et al., 1993). As no reversible label existed for the BTZ binding domain of partially purified Ca^{2+} channels, inhibition of (−)-[^{3}H]devapamil labeling of the PAA binding domain was used to identify benzazepinones with high affinity. Among all benzazepinones tested, Bz-BAZ (an *N*-propyl-substituted benzazepinone, NPSB; for structural formula see Figure 1) was the most potent inhibitor. It carries a hydrophobic phenyl ring distant from the basic amine, an essential pharmacophore on the N^1-ethylamine substituent required for calcium antagonistic activity (Kimball et al., 1992). Bz-BAZ inhibited (−)-[^{3}H]devapamil binding to the partially purified and membrane-bound channel with IC_{50} values of 4.7 ± 2.3 nM (n = 3) and 16 ± 8.5 nM (n = 3), respectively (see Figure 2A). (+)-*cis*-[^{3}H]Diltiazem binding to the membrane-bound channel was inhibited with slightly lower potency (IC_{50} = 35 ± 12.5 nM, n = 4) but still with higher affinity than by (+)-*cis*-diltiazem itself.

Figure 2B illustrates the loss of affinity for (+)-*cis*-diltiazem when the channel is partially purified. A ≈5-fold increase in the IC_{50} for (−)-[^{3}H]devapamil binding inhibition (membranes: IC_{50} = 48–150 nM (n = 2), partially purified: IC_{50} = 520 ± 72 nM, n = 3) was observed. Accordingly, no reversible (+)-*cis*-[^{3}H]diltiazem binding activity was detectable in the partially purified channel preparation (not shown).

To obtain a compound suitable for the direct labeling of the BTZ binding site, we replaced the phenyl group of Bz-BAZ with a fluorescent DMBODIPY moiety. This compound, termed DMBODIPY-BAZ, also retained high affinity for the partially purified channel ((−)-[^{3}H]devapamil binding inhibition: IC_{50} = 33 ± 2.8 nM, n = 3, Figure 2C). Similar to Bz-BAZ, the affinity was lower for the membrane-bound (+)-*cis*-[^{3}H]diltiazem-labeled BTZ binding domain (IC_{50} = 930 ± 310 nM, n = 5). Both benzazepinones partially

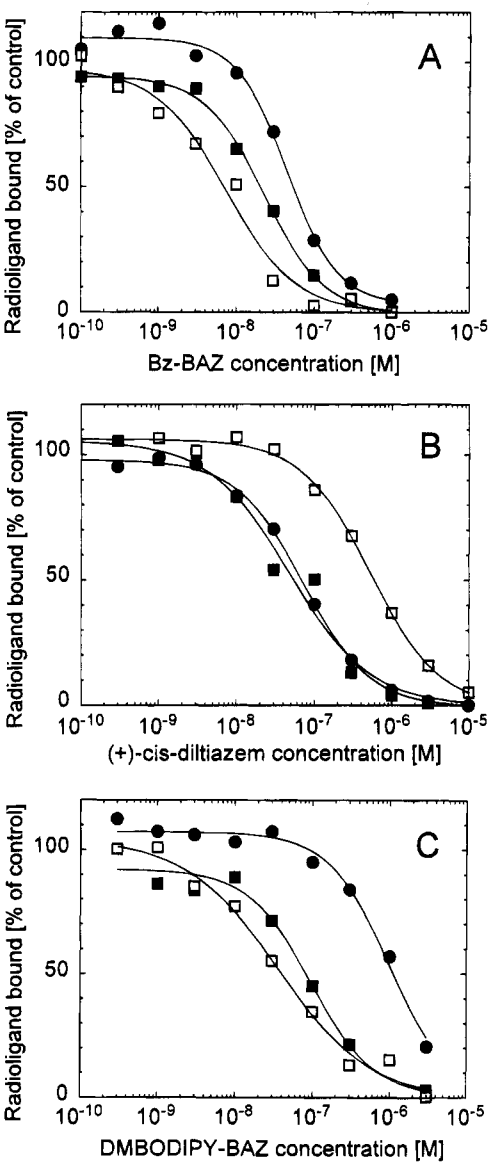

FIGURE 2: Inhibition of (−)-[^{3}H]devapamil and (+)-*cis*-[^{3}H]-diltiazem binding to L-type calcium channels by Bz-BAZ, (+)-*cis*-diltiazem, and DMBODIPY-BAZ. Membrane-bound (filled symbols) and partially purified (open symbols) channel preparations were incubated with (−)-[^{3}H]devapamil (0.5-1.0 nM, 0.014−0.028 mg/mL of membrane protein (■); 0.5−2.0 nM, 0.008−0.010 mg/mL of partially purified channel protein (□)) or (+)-*cis*-[^{3}H]-diltiazem (0.9−3.8 nM, 0.15−0.20 mg/mL of membrane protein (●)) in the presence of increasing concentrations of drugs. Binding is expressed as percent of control binding determined in the absence of drugs. The following binding parameters were obtained by nonlinear curve fitting (given as IC_{50}, slope factor): (A) Inhibition by Bz-BAZ: (●) 44 nM, 1.32; (■) 22 nM, 1.15; (□) 4 nM, 1.14. (B) Inhibition by (+)-*cis*-diltiazem: (●) 72 nM, 1.01; (■) 48 nM, 0.84; (□) 524 nM, 0.97. (C) DMBODIPY-BAZ: (●) 980 nM, 1.10; (■) 95 nM, 1.07; (□) 36 nM, 0.73.

inhibited (+)-[^{3}H]isradipine labeling of native and purified channels (not shown).

Taken together, these experiments suggest that the channel retains its ability to bind the two *N*-propylamino-substituted benzazepinones after purification.

Direct Labeling of the BTZ Binding Domain Using DMBODIPY-BAZ. To directly prove this hypothesis, the binding of DMBODIPY-BAZ to partially purified calcium channel preparations was investigated. Figure 3A shows the fluorescent properties of DMBODIPY-BAZ. As expected for the DMBODIPY fluorophore (Knaus et al., 1992a; Knaus et al., 1992b), the maxima of the excitation and emission spectra were at 511 and 515 nm, respectively. DMBODIPY-BAZ concentrations were routinely determined after direct excitation at 488 nm, measuring emission at 517 nm (see Materials and Methods section). Channel-bound DMBODIPY-BAZ was determined after separation of bound from free ligand using charcoal adsorption. Nonspecific binding was determined by inclusion of an excess of 1 μM Bz-BAZ in the assay mixture. Using this technique, a specific binding component was detected in the partially purified channel preparation, which increased linearly with the protein concentration (0.010−0.080 mg/mL). DMBODIPY-BAZ binding was saturable (Figure 3B) with a dissociation constant of 25 ± 13 nM and a B_{max} of 580 ± 134 pmol/mg (n = 5) of protein. Scatchard transformations of the saturation binding data were linear (r > 0.97, n = 3), indicating the existence of a single population of high-affinity sites. The B_{max} value is about 2−3-fold higher than for other radiolabeled calcium antagonists in the same channel preparations (Berger et al., 1994). Further experiments will be required to determine if this is due to an increase of fluorescence yield upon receptor binding [as previously reported for a DMBODIPY-labeled DHP (Berger et al., 1994)] or a higher binding stoichiometry.

On-Line Detection of DMBODIPY-BAZ Binding Using FRET. Inspection of the excitation spectrum of DMBODIPY-BAZ revealed an overlap with the emission spectrum of the channel protein preparation between 300 and 400 nm (Figure 3A). Within this range, emission is mainly due to the fluorescence of tryptophan residues. This enabled us to develop a fluorescent binding assay that allowed the preferential detection of channel-bound DMBODIPY-BAZ fluorescence. The assay is based on FRET (see Materials and Methods section for experimental details) from tryptophan residues (serving as the energy donors, excited at 285 nm) to the DMBODIPY fluorophore of bound ligand (serving as the energy acceptor). Under conditions of FRET (excitation at 285 nm, emission measured at 517 nm), a saturable fluorescent signal was detected that strictly correlated (Figure 3B,C) to the concentration of specifically bound DMBODIPY-BAZ as determined by the charcoal assay over a wide range of ligand concentration. This correlation was also found when the concentration of specifically bound DMBODIPY-BAZ was decreased by nonfluorescent calcium antagonists (see Figure 5 and below). FRET therefore provides a convenient assay to detect specific binding of DMBODIPY-BAZ to partially purified skeletal muscle calcium channels without the need of separation of bound from free ligand. Measurements are possible at high time resolution and with minimal intra-assay variations. FRET was therefore routinely used to monitor DMBODIPY-BAZ binding kinetics.

Figure 4A shows the association and dissociation kinetics of DMBODIPY-BAZ. Increasing concentrations of DMBODIPY-BAZ (10, 30, 100, and 300 nM) were incubated with 0.02 mg/mL of protein. The time-dependent formation of bound complexes was monitored by FRET until equilibrium was reached. Dissociation of bound DMBODIPY-BAZ was induced by the addition of the unlabeled competitor Bz-BAZ to a final concentration of 1 μM. Association kinetics were monophasic and described by a mono-exponential association reaction (Figure 4A). The apparent association

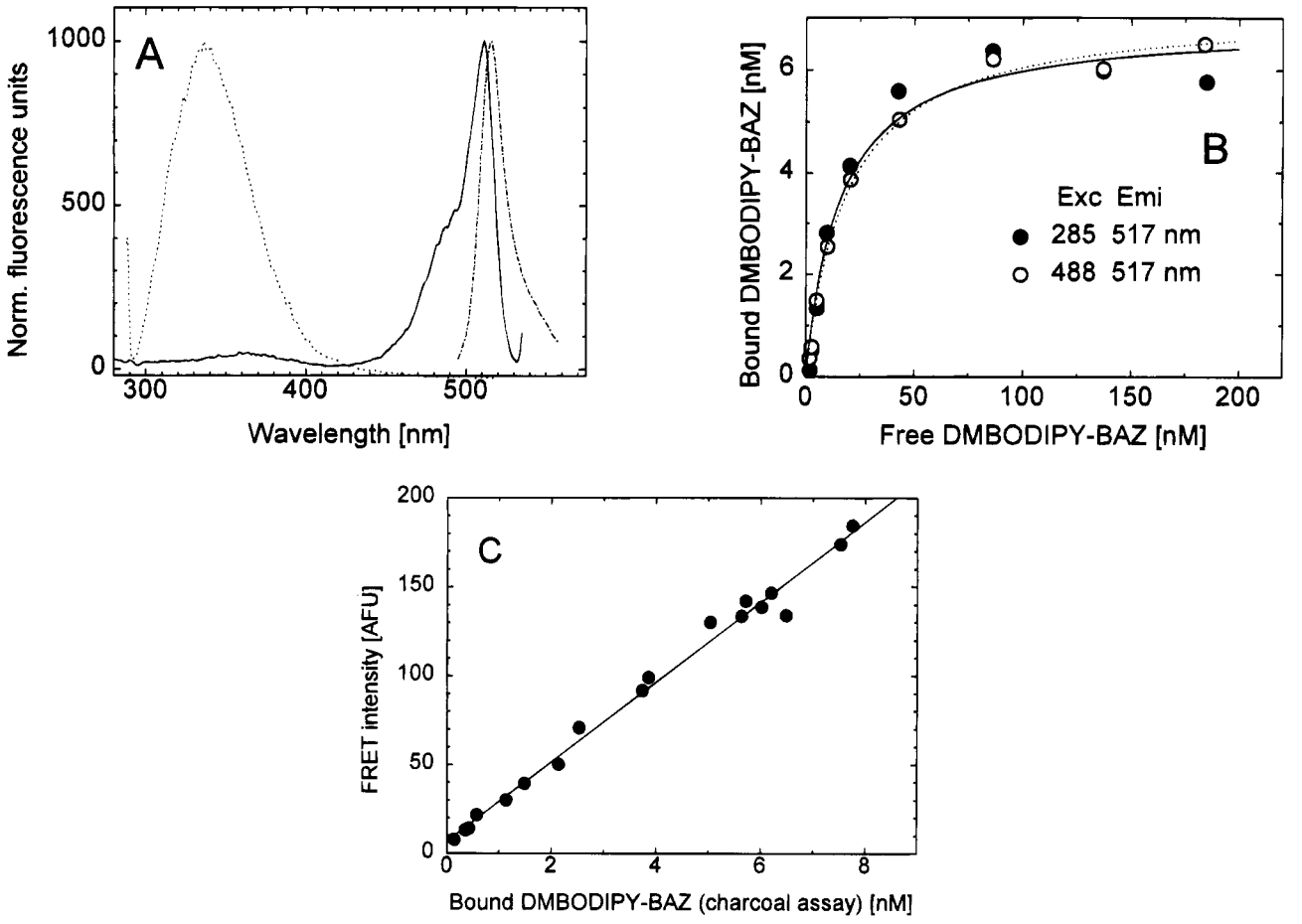

FIGURE 3: (A) Fluorescence spectra of DMBODIPY-BAZ and partially purified receptor. All spectra were recorded in incubation buffer (in the case of receptor: without addition of BSA): (—) excitation spectrum of DMBODIPY-BAZ (100 nM, emission wavelength 540 nm); (— • —) emission spectrum of DMBODIPY-BAZ (100 nM, excitation at 488 nm); (• • •) emission spectrum of partially purified receptor protein (0.02 mg/mL, excitation at 285 nm). Maximal peak intensity was normalized to 1000 AFUs. (B) Saturation analysis. Partially purified calcium channels (0.013 mg/mL of protein) were incubated for 1.5 h with increasing concentrations of DMBODIPY-BAZ. Specific DMBODIPY-BAZ binding was either measured after separation of bound and free ligand (charcoal assay, ○) (excitation (Exc) 488 nm, emission (Emi) 517 nm) or directly using FRET (●) (excitation 285 nm, emission 517 nm). FRET intensity was converted to concentration units using the calibration curve shown in C. The following binding parameters were obtained from nonlinear fits to monophasic saturation isotherms: FRET (solid line): K_d = 15.6 nM, B_{max} = 6.9 nM; charcoal (dotted line): K_d = 18.7 nM, B_{max} = 7.2 nM. (C) Correlation of FRET intensity to bound DMBODIPY-BAZ. FRET intensity was measured in samples from two saturation experiments. The same incubation mixtures were subsequently analyzed for DMBODIPY-BAZ binding using the charcoal assay. FRET intensity is plotted against bound DMBODIPY-BAZ. Linear regression analysis (n = 18, regression coefficient $r > 0.99$) was used to correlate FRET AFUs to the concentration of bound ligand (closed symbols in panel B).

rate constants (k_{app}) were determined by nonlinear curve-fitting (see Materials and Methods section) of the data (Figure 4A). k_{app} values were then plotted against the respective total ligand concentration. The slope of the regression line yielded an association rate constant of $k_{+1} = 6.5 \pm 0.9 \times 10^{-4}$ nM^{-1} min^{-1}, and the y-axis intercept yielded a k_{-1} of 0.055 ± 0.012 min^{-1} for a hypothetical monophasic dissociation. These values are virtually identical to those observed for (+)-*cis*-[^{3}H]diltiazem binding to cardiac sarcolemmal vesicles (Garcia et al., 1986). From the kinetic constants for DMBODIPY-BAZ a K_d of 85 nM was calculated. This value is about 3-fold higher than the K_d derived from equilibrium binding studies. The difference can be explained at least partially: We reproducibly (n = 4) found a slow decay of specific equilibrium binding at DMBODIPY-BAZ concentrations ≥100 nM (Figure 4A). This results in a slight underestimation of maximal specific binding at high DMBODIPY-BAZ concentrations in saturation experiments (Figure 3B) after 90 min of incubation and an underestimating of the apparent K_d value (see legend to Figure 4). Control experiments revealed that this decay was due to neither time-dependent adsorption of ligand or receptor to the cuvette walls nor photobleaching (see Materials and Methods section). Adsorption of channel protein or irreversible loss of binding activity could also be ruled out because it was not observed in incubations containing lower concentrations of DMBODIPY-BAZ. This phenomenon must thus reflect a specific binding mechanism (e.g., negative cooperativity of drug binding at higher ligand concentrations), which is currently investigated in more detail.

A hallmark for diltiazem binding to the BTZ binding domain of membrane-bound L-type calcium channels is the increase of the dissociation rate with the concentration of unlabeled ligand used to block the forward reaction (Prinz & Striessnig, 1993). This phenomenon has been termed "ligand-induced accelerated dissociation" (Prinz & Striessnig, 1993). As shown in Figure 4B an identical behavior was found for DMBODIPY-BAZ binding to the partially purified channel. The dissociation half-life decreased about 9-fold with increasing concentrations of applied Bz-BAZ from 28

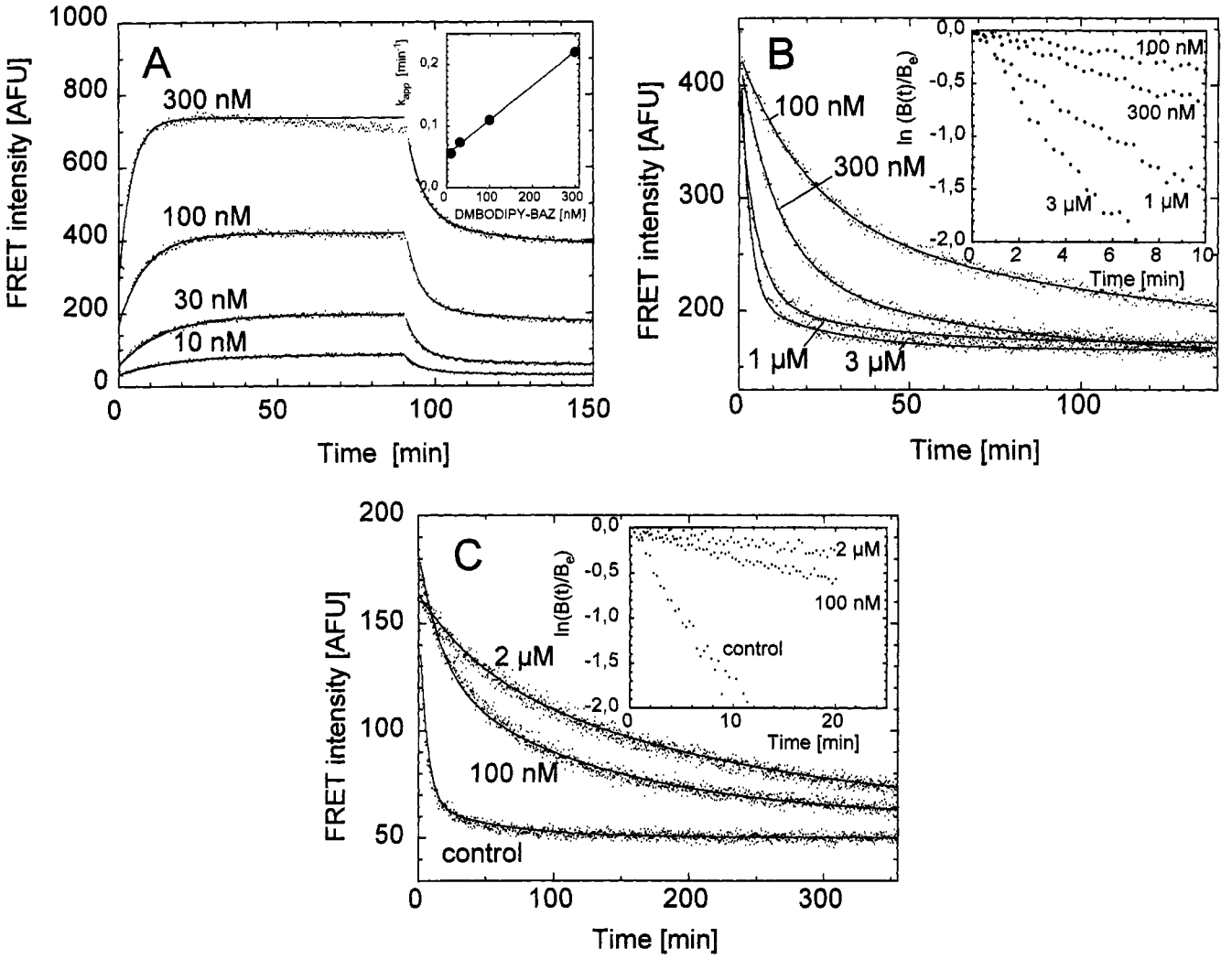

FIGURE 4: Binding kinetics of DMBODIPY-BAZ determined by FRET measurements. Emission: 517 nm; excitation; 285 nm; 1 data point collected every 7−8 s. (A) Four samples containing 0.02 mg/mL of partially purified Ca^{2+} channel protein were simultaneously incubated with different concentrations of DMBODIPY-BAZ in four magnetically stirred cuvettes. Final DMBODIPY-BAZ concentrations were 10, 30, 100, and 300 nM as indicated. After 90 min, the dissociation was induced by the addition of Bz-BAZ to a final concentration of 1 μM. Association reactions were fitted (solid line) by a mono-exponential association curve to determine k_{app}. The following k_{app} values were calculated: 10 nM, 0.056; 30 nM, 0.074; 100 nM, 0.11; 300 nM, 0.22 min^{-1}. Dissociation kinetics were fitted (solid line) to a biexponential dissociation reaction yielding the following parameters (given as k_{-1} fast component/k_{-1} slow component, percent of slow component): 10 nM, 0.23/0.053 min^{-1}, 24%; 30 nM, 0.24/0.036 min^{-1}, 24%; 100 nM, 0.24/0.028 min^{-1}, 21%; 300 nM, 0.20/0.038 min^{-1}, 29%. Binding isotherms were constructed from the specific binding data using either maximal specific binding or specific binding after 90 min of incubation. The K_d values obtained were 56 and 46 nM, respectively. (Inset) Linear regression of a plot of k_{app} versus ligand concentration yields a k_{+1} of 6.5 ± 0.9 × 10^{-4} nM^{-1} min^{-1} and a k_{-1} of 0.055 ± 0.012 min^{-1} (means from $n = 4$ independent experiments). (B) Dissociation induced by Bz-BAZ. After reaching binding equilibrium (0.02 mg/mL partially purified channel protein; 100 nM DMBODIPY-BAZ), dissociation was induced by the addition of Bz-BAZ (final concentrations as indicated: 100 nM, 300 nM, 1 μM, and 3 μM). The following dissociation rate constants k_{-1} were determined by nonlinear curve fitting (given as in panel A): 100 nM, 0.049/0.0085 min^{-1}, 45%; 300 nM, 0.092/0.015 min^{-1}, 32%; 1 μM, 0.21/0.029 min^{-1}, 20%; 3 μM, 0.39/0.040 min^{-1}, 21%. (Inset) Semilogarithmic plots of the dissociation data. (C) Dissociation in the presence of different concentrations of (+)-isradipine. After binding equilibrium (0.02 mg/mL of protein, 20 nM DMBODIPY-BAZ) was reached, (+)-isradipine was added to final concentrations of 2 μM or 100 nM, which caused less than 10% of complex dissociation during 60 min. Control samples received vehicle alone (3 μL of DMSO). After 60 min, 1 μM Bz-BAZ was added to induce dissociation ($t = 0$ min). The following k_{-1} values were determined by nonlinear curve fitting (given as in panel A): control, 0.18/0.018 min^{-1}, 19%; 100 nM (+)-isradipine present, 0.057/0.0078 min^{-1}, 55%; 2 μM (+)-isradipine present, 0.022/0.0043 min^{-1}, 71%. (Inset) Semilogarithmic replot of the dissociation data.

min (100 nM Bz-BAZ) to 3.1 min (3 μM Bz-BAZ). In contrast, inclusion of the DHP (+)-isradipine during the dissociation process caused a dramatic decrease of DMBODIPY-BAZ dissociation in a concentration-dependent manner (Figure 4C). Noncompetitive interaction of DHPs with a slowing of ligand dissociation is also a typical feature of BTZ binding (Garcia et al., 1986; Glossmann et al., 1983). As observed for other classes of calcium antagonists (Reynolds et al., 1986; Berger et al., 1994; Goll et al., 1984), dissociation was biphasic. In the presence of 1 μM Bz-BAZ, the slowly dissociating component accounted for less than about 25% of the dissociation reaction (for time constants see legend to Figure 4A−C). Both the fast and slow components of dissociation were accelerated by increasing concentrations of Bz-BAZ (see legend to Figure 4B) whereas the presence of (+)-isradipine caused the opposite effect (see legend to Figure 4C).

Pharmacological Properties of DMBODIPY-BAZ Binding. The modulation of DMBODIPY-BAZ binding by different chemical classes of calcium antagonists is shown in Figure

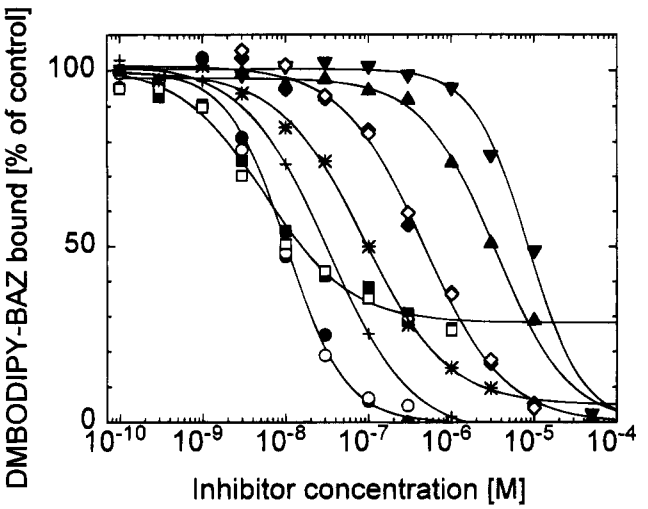

FIGURE 5: Pharmacological profile of DMBODIPY-BAZ binding. Inhibition of DMBODIPY-BAZ (15−20 nM) equilibrium binding by unlabeled calcium antagonists to partially purified channel protein (0.015−0.025 mg/mL) was determined by FRET (closed symbols). Results from the charcoal assay (open symbols) are shown as a comparison for three drugs. The following binding parameters were determined using nonlinear curve fitting of the data to the general dose−response equation (given as IC_{50}, slope factor): Bz-BAZ/FRET (●), 10 nM, 1.25; Bz-BAZ/charcoal (○), 9 nM, 1.16; (+)-*cis*-diltiazem (▲), 3.4 μM, 0.99; (−)-*cis*-diltiazem (▼), 8.4 μM, 1.34; (+)-tetrandrine/FRET (♦), 460 nM, 0.89; (+)-tetrandrine/charcoal (◊), 430 nM, 0.87; (+)-isradipine/FRET (■), 5.6 nM, 0.85; (+)-isradipine/charcoal (□), 5.2 nM, 0.89; (−)-devapamil (*), 86 nM, 0.86; Tinuvin 770 (+), 32 nM, 0.87.

5. Bz-BAZ (IC_{50} = 12 ± 6.0 nM, n = 4) was the most potent inhibitor. The loss of affinity of the purified channel for (+)-*cis*-diltiazem was evident from the high IC_{50} values for DMBODIPY-BAZ binding inhibition [(+)-*cis*-diltiazem: IC_{50} = 3500 ± 100 nM, K_i = 2060 nM; (−)-*cis*-diltiazem: IC_{50} = 9500 ± 710 nM, n = 3). Bz-BAZ was about 300-fold more potent than (+)-*cis*-diltiazem and about 50-fold more potent than (+)-tetrandrine. (+)-Tetrandrine and Tinuvin 770 are non-BTZ-related drugs that competitively bind to the BTZ binding domain in cardiac and skeletal muscle plasma membranes (King et al., 1988; Glossmann et al., 1993). PAAs completely blocked binding of DMBODIPY-BAZ whereas the DHP (+)-isradipine caused incomplete inhibition at 25 °C (IC_{50} = 7.5 ± 3.5 nM, maximal inhibition to 31 ± 4% of control binding, n = 3). We were unable to test for the potential stimulatory effects of (+)-isradipine at 37 °C because of the rapid irreversible inactivation of calcium antagonist binding observed at this temperature (Berger et al., 1994; Schneider et al., 1991).

DMBODIPY-BAZ Is a Potent Blocker of L-Type Calcium Channels in A7r5 Cells. In order to compare the calcium channel blocking properties of DMBODIPY-BAZ with those of (+)-*cis*-diltiazem, we measured their effects on L-type calcium channels in A7r5 cells using the whole cell patch−clamp technique. A7r5 cells are derived from smooth muscle cells and express smooth muscle L-type calcium currents (Hering et al., 1993). As the channel block by diltiazem-like calcium antagonists is channel state-dependent (Lee & Tsien, 1983), we distinguished two components of calcium current inhibition: "Tonic" block was observed as current inhibition during the first test pulse after incubation of the cell with drug for 2 min. Tonic block reflects drug interaction with the resting channel. Additional "use-dependent" current inhibition occurred during repetitive depolarization (400 ms test pulses, 0.1 Hz) of the cell membrane and reflects preferential drug binding to the open and/or inactivated channel. Figure 6 illustrates the blocking effects of 10 μM DMBODIPY-BAZ and (+)-*cis*-diltiazem under identical experimental conditions. The extent of use-dependent block of peak I_{Sr} after the pulse train by both compounds was similar for both drugs (82 ± 11% for (+)-*cis*-diltiazem, n = 5; 70 ± 10% for DMBODIPY-BAZ, n = 3) as was the extent of initial block of peak I_{Sr} (29 ± 6% for (+)-*cis*-diltiazem, n = 4; 22 ± 5% for DMBODIPY-BAZ, n = 3). Both drugs accelerated current inactivation kinetics during the test pulse. No shift of the maximum of the current voltage relationship was observed for both drugs (not shown). Maximal inhibition at 1 μM concentrations was 75 ± 9% and 51 ± 8% by (+)-*cis*-diltiazem and DMBODIPY-BAZ, respectively, with values for the initial block of 15 ± 3% (n = 6) and 12 ± 4% (n = 4).

A slower onset of use-dependent block was found for DMBODIPY-BAZ requiring about two to three times longer for half-maximal block to develop than (+)-*cis*-diltiazem (see legend to Figure 6). Recovery from block at negative holding potentials (5 min rest at −70 mV) was also less complete for DMBODIPY-BAZ. After a block by 10 μM (+)-*cis*-diltiazem, 65 ± 7% (n = 4) of the control current were recovered. In contrast, only insignificant recovery (8 ± 4% of control current, n = 3), was found for DMBODIPY-BAZ during the same period of time. Recovery was only slightly enhanced by additional washing of the cell with Sr^{2+} solution (recovery to 14 ± 4%, n = 3, of control current).

Taken together, the calcium channel blocking effects of DMBODIPY-BAZ closely resemble those of (+)-*cis*-diltiazem. However, development of and recovery from block are slower for DMBODIPY-BAZ.

DISCUSSION

We synthesized NPSBs as a new series of diltiazem analogues. They retain high affinity for partially purified L-type calcium channels and (as shown for DMBODIPY-BAZ) are at least as potent in blocking currents through smooth muscle L-type calcium channels as is (+)-*cis*-diltiazem. The fluorescent DMBODIPY-BAZ was synthesized as a unique tool to characterize the BTZ binding domain of purified calcium channels and exploit fluorescence resonance energy transfer to develop a highly time-resolved binding assay.

Using DMBODIPY-BAZ as a probe, we could address the question if the BTZ binding domain exists on partially purified L-type calcium channels. Such studies have been impossible so far because of the dramatic loss of binding affinity for (+)-*cis*-[^{3}H]diltiazem, the only available radioligand for the BTZ binding domain (Garcia et al., 1986). Previous studies could only indirectly infer the existence of a binding site for BTZ on the purified channel by either photoaffinity labeling (Striessnig et al., 1990; Naito et al., 1989) or by measuring the noncompetitive (Striessnig et al., 1986; Flockerzi et al., 1986) effects of unlabeled BTZs on the two other well-characterized binding domains (for DHPs and PAAs). The latter approach was taken to first search for diltiazem analogues that retain (or gain) high affinity for the purified channel. From extensive SAR studies of diltiazem-related benzazepinones (Kimball et al., 1992), it

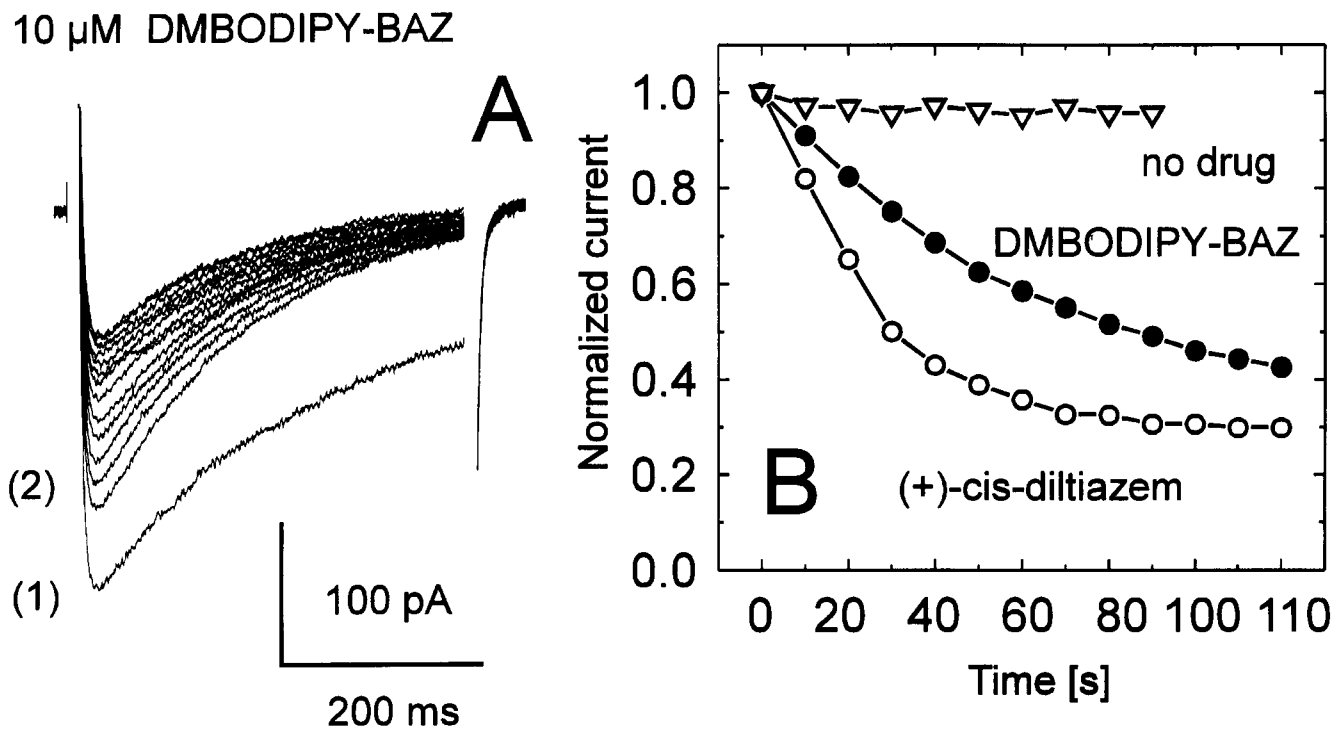

FIGURE 6: (A) I_{Sr} was evoked by 400-ms pulses from −70 to +30 mV with an interpulse interval of 10 s. Inhibition of I_{Sr} during the train 2 min after bath application of 10 μM DMBODIPY-BAZ is shown: (1) the control current before drug application and (2) the first current during the pulse train in the presence of drug. (B) To observe the kinetics of use-dependent block by (+)-*cis*-diltiazem and DMBODIPY-BAZ, normalized peak I_{Sr} from the experiment shown in panel A was plotted against the number of pulses: (▽) control peak currents before drug application, (○) after the addition of 10 μM (+)-*cis*-diltiazem, and (●) after the addition of 10 μM DMBODIPY-BAZ to an other cell (peak currents from traces in panel A). Time for development of maximal current inhibition was 30 ± 12 s (n = 3) and 84 ± 20 s (n = 3) for (+)-*cis*-diltiazem and DMBODIPY-BAZ, respectively.

is known that the introduction of bulky substituents distant from the basic amine pharmacophore does not cause a major reduction of calcium antagonist affinity (Kimball et al., 1992). The benzazepinone precursor (**2b**) contains a free amino group and is suitable for the introduction of radiolabeled or fluorescent moieties. Introduction of even bulky substituents like a phenyl ring (Bz-BAZ) or fluorescent DMBODIPY (DMBODIPY-BAZ) yielded compounds with high affinity for the partially purified channel. This was proven by employing DMBODIPY-BAZ in direct labeling experiments, both by conventional separation techniques and by FRET.

DMBODIPY-BAZ binding studies confirmed the low affinity of the purified channel for (+)-*cis*-diltiazem. Nevertheless, the binding site retained the most important properties previously described for (+)-*cis*-[^{3}H]diltiazem binding in different tissues: As shown for (+)-*cis*-[^{3}H]-diltiazem binding in membranes (Garcia et al., 1986; Glossmann et al., 1983), DHPs partially inhibited DMBODIPY-BAZ binding at 25 °C and dramatically slowed dissociation of the ligand−receptor complex. We also observed the increase of the dissociation rate constant with the concentration of unlabeled ligand (ligand-induced accelerated dissociation) typical for dissociation from the BTZ binding domain (Prinz & Striessnig, 1993). We conclude that the DMBODIPY-BAZ labeled site on the partially purified calcium channel can be classified as a BTZ binding domain with most of the properties described for membrane-bound channels.

Two of the binding properties of the newly synthesized benzazepinones were unexpected. We have no clear explanation for their lower binding affinity for the (+)-*cis*-[^{3}H]-diltiazem labeled channel when membrane bound. As this was more pronounced for DMBODIPY-BAZ (Figure 3B) than for Bz-BAZ, the bulky DMBODIPY side chain may be responsible for this phenomenon. The side chain could slow its optimal coordination within the binding domain, the effect being more pronounced in the membrane-bound state. Alternatively, purification may lead to discrete conformational changes of the channel's α_1-subunit exposing additional interaction sites for DMBODIPY-BAZ. The high time resolution and low intra-assay variation of the FRET technique also allowed us to detect complex association and dissociation reactions. In the presence of high (≥100 nM) ligand concentrations, monophasic association was followed by a decrease of the specific binding signal (Figure 4A). As nonspecific effects (see above) can be ruled out, this phenomenon is most likely due to a specific binding mechanism. High ligand concentrations could promote the slow rearrangement to a different conformational state of the complex, resulting in changes of binding affinity and/or of the fluorescent properties of the ligand [like changes in fluorescence yield (Berger et al., 1994)]. This would also account for the biphasic dissociation reaction observed when the forward reaction was blocked with an unlabeled competitor. We have recently found a slow monophasic association combined with a biphasic dissociation reaction for DHP binding to purified calcium channels (Berger et al., 1994). Quantitative analysis of the data in terms of a specific binding scheme revealed that this could simply be explained by considering one or more intermediate conformational states for the formation of the DHP−receptor complex. We therefore suggest that a similar stepwise binding reaction accounts for the kinetic data observed with DMBODIPY-

BAZ. Further experiments are required to confirm this hypothesis.

The FRET assay will also enable further investigation of the complex noncompetitive binding interaction between BTZs and DHPs (as also described in this paper in Figure 4C), addressing the question if steric or allosteric interactions account for the slowing of DMBODIPY-BAZ dissociation by DHPs. This differentiation will have important structural implications as the demonstration of steric interactions would place the DHP and BTZ binding domains in close proximity on the extracellular channel surface (Hering et al., 1993).

Taken together, our studies demonstrate that the BTZ binding domain can be labeled now even on partially purified L-type calcium channels. Reversible and photoaffinity labeling studies employing appropriately substituted benzazepinones, like, for example, photoreactive or fluorescent derivatives of **2b**, should therefore represent the ideal experimental approach to provide further insight into the molecular architecture of the BTZ binding domain on L-type calcium channels.

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