A Unique Fluorescent Phenylalkylamine Probe for L-type Ca²⁺ Channels

COUPLING OF PHENYLALKYLAMINE RECEPTORS TO Ca²⁺ AND DIHYDROPYRIDINE BINDING SITES

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The first fluorescently labeled phenylalkylamine, DMBODIPY-PAA (5-[3-[[4,4-difluoro-5,7-dimethyl-3a,4-diaza-4-bora-indacen-3-yl]propionamido]phenethyl-N-methylamino]-2-isopropyl-2-(3,4,5-trimethoxyphenyl)valeronitrile) has been introduced for L-type Ca²⁺ channel research. DMBODIPY-PAA binds reversibly to L-type Ca²⁺ channels purified from rabbit skeletal muscle microsomes by wheat germ agglutinin-Sepharose chromatography. In this preparation DMBODIPY-PAA labels 412 pmol of phenylalkylamine receptors/mg of protein with a Kₐ of 6.82 nM and a favorable signal-to-noise ratio. Therefore DMBODIPY-PAA has a higher affinity for purified Ca²⁺ channels than the commonly employed radioligands and consequently has assisted in channel purification after prelabeling by simply monitoring receptor-bound fluorescence. (+)-PN200-110 (which is stimulatory for (+)-[3H]desmethoxyverapamil binding to purified Ca²⁺ channels) inhibits DMBODIPY-PAA labeling. Since these drug interactions are reciprocal, the phenylalkylamine and dihydropyridine binding sites of the α₁-subunit are tightly coupled. Kinetic and equilibrium binding studies with (+)-[3H]desmethoxyverapamil and DMBODIPY-PAA show that phenylalkylamine binding to L-type Ca²⁺ channels is dependent on Ca²⁺. Chelation of divalent metal ions converts phenylalkylamine receptors into a very low affinity state. This conversion is temperature- and time-dependent and completely reversible (Kₐ = 58 nM). This study demonstrates the utility of fluorescent ligands for binding studies with L-type Ca²⁺ channels and provides evidence for coupling between Ca²⁺ binding sites and phenylalkylamine receptors.

L-type Ca²⁺ channels mediate voltage-controlled Ca²⁺ entry into a variety of excitable cells (1). The activity of these channels is modulated by different classes of drugs (dihydropyridines, benzothiazepines, diphenylbutylpiperidines, phenylalkylamines, piperazinylindoles, amiloride analogs, benzothiazinones), commonly known as Ca²⁺ channel antagonists (for reviews see Refs. 2-4 and 5-7). The effects of these drugs are exerted by binding to allosterically coupled domains on the pore forming α₁-subunit (4, 8, 9). L-type Ca²⁺ channels can be purified from mammalian skeletal muscle membranes as a complex consisting of α₁-, β₁-, γ₁-, and α₂δ-subunits (10-12) and can be functionally reconstituted (9, 13). This isolated complex is the only purified Ca²⁺ channel preparation where direct equilibrium binding studies with radiolabeled Ca²⁺ channel ligands have been reported (9, 13, 14).

Radiolabeled phenylalkylamines ([±]-[3H]gallipamil, ([±]-[3H]verapamil, ([±]-[3H]deshemtoxyverapamil, [N-methyl-3H]LU49888) are useful ligands to investigate the properties of L-type Ca²⁺ channels in neuronal (15, 16), heart (17), and skeletal muscle transverse tubule membranes (16, 18-20). Purified skeletal muscle L-type Ca²⁺ channels were recently used to identify the phenylalkylamine binding region within the α₁-subunit, after photolabeling with [N-methyl-3H]LU49888 (21). This was possible despite a greater than 10-fold loss of ligand affinity with respect to the membrane-bound state (22). The phenylalkylamine receptor site is located between Glu-1349 and Trp-1391 (21) (numbering refers to the deduced amino acid sequence reported by (23)). This binding site includes the S6 segment of domain IV (see Refs. 24 and 24 for discussion) and the beginning of a long evolutionary highly conserved cytoplasmic region (25) which contains a putative Ca²⁺ binding domain, predicted by the Tuftkroxtingen test (26). This observation is further supported by the finding that phenylalkylamine interaction with membrane-bound L type Ca²⁺ channels is always inhibited by divalent cations (e.g. Ca²⁺ and Cd²⁺) (see Refs. 2 and 4 for reviews). In contrast, dihydropyridines require bound Ca²⁺ for high-affinity binding (2, 4, 27, 28). Furthermore the diphenylbutylpiperidinyl fluspirilene binds with increased affinity to L-type Ca²⁺ channels in sarcolemmal membranes, which ions which block the channel (Cd²⁺, Co²⁺, and Ni²⁺) are present. Ions which pass the channel easily (Ba²⁺, Sr²⁺, and Ca²⁺) inhibit fluspirilene binding (29). Taken together these find-

The abbreviations used are: LU49888, (S)-5-[(3-azidophenethyl)-(N-methyl)methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile; EGTG, [ethylenebis(oxyethylenenitrilo)tetraacetic acid]; BM 20.1140, ethyl-2,2-diphenyl-4-(1-pyridolinio)-5-[2-picolyl]oxycarboxylic acid; BSA, bovine serum albumin; DMBODIPY™ (a trademark of Molecular Probes, Inc.), 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-indacene-9-propionic acid; D600, dimethoxyverapamil; D619, (±)-2-methyl-3-cyano-3-(3',4'-dimethoxyphenyl)-2-isopropylvaleronitrile; D888, desmethoxyverapamil; PAA, phenylalkylamine; PN200-110, isopropyl-4-(2,3,5-benzoazolazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-ethoxycarbonylpyridine-3-carboxylate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.
ings point to the existence of different conformations of the α₁-subunit in the nonconducting (depolaredized) state prevalent in isolated membranes. These conformations are apparently stabilized by ions and drugs but are far from being understood at the molecular level.

L-type Ca²⁺ channels are unique among voltage-regulated cation channels as their physiological ion selectivity is maintained by their ability to bind Ca²⁺ and thereby exclude the far more abundant monovalent cations (30–33). If Ca²⁺ is removed experimentally by chelators, the channel is highly permeable for Na⁺. In vertebrate skeletal muscle transverse tubular membranes the α₁-subunit is also part of a unique transmembrane communication pathway. In this system α₁ acts as a voltage sensor and controls the Ca²⁺ release from the ryanodine receptor/foot structure (34). The voltage sensor function of the α₁-subunit has an absolute requirement for extracellular divalent metal ions (Ca²⁺ > Sr²⁺ > Mg²⁺ > Ba²⁺). The requirement can also be fulfilled by monovalents (Li⁺ > Na⁺ > K⁺ > Rb > Cs) but not by organic divalents (dime-thonium) or monovalents (tetraethylammonium) (34, 35).

The essential metal ion binding sites for excitation-contraction coupling has been termed the “priming site.” Relative affinities for cations to this priming site are similar to the relative permeability ratios of L-type cardiac Ca²⁺ channels. For example Ca²⁺ is 1,000-fold more effective than Na⁺ for priming site binding and Ca²⁺ channel permeation. The regions of the Ca²⁺ channel which participate in Ca²⁺ binding (and are essential for ion transport as well as voltage sensor function) have not yet been identified. Evidence that the dihydropyridine receptor region is connected to, or even forms part of, such a high affinity Ca²⁺ binding site was recently presented (27).

In the course of an investigation on the coupling of dihydropyridine receptors to Ca²⁺ binding sites and the mechanism of positive heterotropic allosteric regulators (27), we observed that phenylalkylamine binding to purified Ca²⁺ channels from rabbit skeletal muscle was inhibited by chelators (EGTA and EDTA) and highly dependent on free Ca²⁺. Despite the availability of reasonably pure and stable Ca²⁺ channel preparations since many years (36) and recent advances in the reconstitution of drug binding sites (37) as well as functional reconstitution experiments (9, 13), the kinetic properties of Ca²⁺ channels have remained unexplored. Advantages of the system are that sidelessness problems (see Refs. 28, 38, and 39) in contrast to the membrane-bound state are absent. Such studies could eventually lead to a deeper understanding of the molecular mechanisms which govern the interactions between Ca²⁺, the Ca²⁺ channel complex, and drugs and bridge the gap between functional and structural approaches.

We synthesized several fluorescent phenylalkylamines, including DMBODIPY-PAA. DMBODIPY-PAA can be employed to label L-type Ca²⁺ channels directly by measuring channel-bound fluorescence in a spectrofluorometer. We therefore had the unique opportunity to probe Ca²⁺ channel-linked phenylalkylamine receptors with both a radiolabeled and a fluorescent probe which are structurally different. In the following the properties of DMBODIPY-PAA and observations on the coupling of Ca²⁺ binding and dihydropyridine sites with the phenylalkylamine binding domain are reported.

**MATERIALS AND METHODS**

_D Fluorescent Phenylalkylamine and L-type Ca²⁺ Channels_
branes (98.6 mg of protein) were solubilized by gently stirring with 1% digitonin (w/v) in 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM iodoacetamide, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A (buffer C) for 45 min at 2 °C, with the protein/detergent ratio kept at 1:5. After centrifugation for 60 min at 4 °C (60,000 × g, Sorvall SS-34 rotor), the supernatant (43 ml) was incubated with 14.3 nmol of DMBODIPY-PAA for 15 min at 37 °C. Prior to chromatography on WGA-Sepharose, the mixture was cooled down to 2 °C. Chromatography was then performed as described in Ref. 43. The 5% (w/v) N-acetylgalactosamine-eluted fractions were layered onto a 40-ml 5-20% (w/w) continuous sucrose density gradient in buffer C (without NaCl) and centrifuged at 50,000 rpm for 105 min at 2 °C (Beckman rotor VT50). After the purification steps, the fluorescence of DMBODIPY-PAA and, in parallel, specific binding of (+) [3H]PN200-110 were determined.

**Protein Determination and Data Analysis**—Protein of the particulate membrane preparations was determined by the method of Lowry et al. (46) and of purified skeletal muscle Ca2+ channels according to Bradford (40) with BSA as standard. Binding inhibition curves were parameter-optimized using the general dose-response equation (47) and the program package GraphPAD-InPlot® version 3.14 (GraphPad Software Inc.). The IC50 and EC50 values are the drug concentrations causing 50% inhibition or stimulation, respectively. Inhibition (in percent) is defined as 100 × (B - B0)/B0, where B is specific binding of the labeled ligand in the presence and B0, in the absence of added drug. Stimulation is defined as (B0/B) × 100. Dissociation kinetics were fitted by nonlinear methods and association data were linearized as recently described (27).

**RESULTS**

**General Properties of DMBODIPY-PAA**—The excitation-emission spectra for DMBODIPY-PAA in methanol are shown in Fig 1. The fluorescence emission was highly sensitive to the environment as was observed with other fluorescent ligands (48). Digitonin stimulated DMBODIPY-PAA fluorescence, in comparison with buffer A without detergent. Enhancement by digitonin was maximal (8.5-fold compared with buffer A) between 0.08 and 0.1% (w/v), and half-maximal stimulation was observed at 0.012% (w/v) digitonin. Compared with methanol, the maximum emission in digitonin buffer was shifted from 511 to 517 nm (not shown).

The interaction of DMBODIPY-PAA with L-type Ca2+ channel-linked phenylalkylamine receptors was first investigated in displacement experiments as summarized in Table I. DMBODIPY-PAA, in contrast with (±)-desmethoxyverapamil, is less potent than the standard phenylalkylamine in neuronal and skeletal muscle membranes. To our surprise purification of the L-type Ca2+ channel from skeletal muscle increases the affinity for DMBODIPY-PAA about 2-fold, whereas (±)-desmethoxyverapamil, (−-[3H]desmethoxyverapamil (14), and [N-methyl-3H]LU49888 (43) suffer decreases in affinity. (+)-PN200-110 (1 μM) when added to purified Ca2+ channels decreased DMBODIPY-PAA’s affinity (∼3-fold) (not shown) for the phenylalkylamine receptors, but increased the affinity for (−-[3H]desmethoxyverapamil (14).

The allosteric interaction of DMBODIPY-PAA with the dihydropyridine sites was investigated in guinea pig brain membranes and with purified Ca2+ channels. In guinea pig brain membranes, DMBODIPY-PAA inhibited (+) [3H]PN200-110 binding only partially (maximal inhibition 82% at 10 μM, IC50 value: 21 nM). DMBODIPY-PAA inhibited (+) [3H]PN200-110 binding to purified Ca2+ channels completely with an IC50 value of 18 nM and a pseudo Hill slope of 0.74. In contrast, (−)-desmethoxyverapamil stimulated dihydropyridine binding in this preparation as previously reported (14). As will be shown in the section below, these allosteric interactions between phenylalkylamine and dihydropyridine sites are reciprocal.

**DMBODIPY-PAA Binding to Purified L-type Ca2+ Channels**—The increase in affinity of DMBODIPY-PAA, which occurred when channels were purified (see above), and the enhancement of fluorescence in digitonin buffer prompted us to study the interaction of the ligand with purified Ca2+ channels directly by quantitation of channel-bound fluorescence. To this end a charcoal adsorption assay was employed to separate unbound DMBODIPY-PAA from Ca2+ channel-bound label (see “Materials and Methods”). Channel-bound as well as total ligand concentrations were then determined from a standard curve constructed in buffer B. This standard curve was linear between 0.1 and 50 nM DMBODIPY-PAA (γ = arbitrary fluorescence units × 0.06484, where γ is DMBODIPY-PAA in nanomolar; r = 0.999) and was not changed by the presence of Ca2+ channels (not shown). In order to estimate the dissociation of ligand during the separation step, ice-cold charcoal adsorption medium was added to assays containing Ca2+ channels with bound DMBODIPY-PAA and left up to 60 min at 0 °C before separation by centrifugation. The data for bound ligand obtained at the different time points were fitted by a straight line. The slope of this line indicated a 0.1% loss of bound label per minute. Since the entire separation method requires 5 min, our data underestimate bound label by <1%.

**Fig. 1. Structure and spectral characteristics of DMBODIPY-PAA.** The excitation (dashed line) and emission (solid line) spectra of 250 nM DMBODIPY-PAA, dissolved in methanol, is shown. The slit width for both spectra was 4 nm. The inset shows the structure of DMBODIPY-PAA composed of the phenylalkylamine precursor (above) and the fluorophore DMBODIPY* (below). The asterisk indicates the asymmetric carbon atom in the phenylalkylamine part of the molecule.
Fluorescent Phenylalkylamine and L-type Ca\textsuperscript{2+} Channels

TABLE I

<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+} channel preparation</th>
<th>DMBODIPY-PAA</th>
<th>(\pm)-Desmethoxyverapamil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50}</td>
<td>nM</td>
</tr>
<tr>
<td>Cerebral cortex membranes</td>
<td>49.6 ± 21.5 (3)</td>
<td>0.75 ± 0.12</td>
</tr>
<tr>
<td>Skeletal muscle membranes</td>
<td>21.6 ± 11.7 (3)</td>
<td>1.03 ± 0.16</td>
</tr>
<tr>
<td>Purified Ca\textsuperscript{2+} channels</td>
<td>10.9 ± 1.2 (4)</td>
<td>0.92 ± 0.23</td>
</tr>
</tbody>
</table>

FIG. 2. Binding properties of DMBODIPY-PAA to purified rabbit skeletal muscle L-type Ca\textsuperscript{2+} channels. A, ligand saturation studies with receptors. Purified Ca\textsuperscript{2+} channel protein (0.0027–0.171 mg/ml corresponding to 1–62.5 nM phenylalkylamine receptor concentration; 366 pmol/mg of protein) were incubated with 2.28 nM DMBODIPY-PAA for 30 min at 22 °C in a final assay volume of 2 ml. After charcoal separation of unbound ligand and determination of specifically bound ligand, data (means from duplicates) are expressed in a Scatchard plot. Plotted are the ratios of bound to free receptor concentrations (where free receptor was calculated from total minus bound receptor) against bound ligand concentration which equals bound ligand concentration (see Ref. 49 for details). The x axis intercept of 1.16 nM corresponds to a binding ability of ≈51%; K\textsubscript{d} (–1/slope) = 5.64 nM; r = 0.974. B, receptor saturation studies with ligand. DMBODIPY-PAA (0.162–19.405 nM) was incubated with 0.0125 mg/ml purified Ca\textsuperscript{2+} channel protein for 30 min at 22 °C in a final assay volume of 2 ml. 0, total binding; 0, binding in the presence of (+)-desmethoxyverapamil; 0, specific binding. K\textsubscript{d} = 4.58 ± 0.13 nM; B\textsubscript{max} = 3.24 ± 0.25 nM which corresponds to 366 pmol/mg of protein. A binding ability of 50% (see above) was taken into account for the calculation of free DMBODIPY-PAA. Means from duplicate determinations. C, Hofstee transformation of the equilibrium binding data presented in B, D, association kinetics. DMBODIPY-PAA (4.11 nM bindable enantiomer) was incubated for the indicated times with 0.0119 mg/ml of purified Ca\textsuperscript{2+} channel protein (3.9 nM of phenylalkylamine receptors) in a final assay volume of 2 ml. The inset shows a semilogarithmic transformation of the data according to the second-order rate equation (see Ref. 27). k\textsubscript{+} = 0.305 ± 0.017 min\textsuperscript{–1}. k\textsubscript{–} = 0.12 ± 0.004 min\textsuperscript{–1}. The inset shows a semilogarithmic transformation of the data. B, the specifically bound ligand at time t after initiation of dissociation (for mean values, see Table II).
DMBODIPY-PAA. At 22 °C, the association rate constant was 0.302 ± 0.017 nM⁻¹·min⁻¹. Dissociation of radiolabeled phenylalkylamines from L-type Ca²⁺ channels can occur in a monophasic (6, 18, 20, 50) or biphasic manner (16, 51).

At 22 °C the dissociation of DMBODIPY-PAA and of (-)-[³H]desmethoxyverapamil, initiated by addition of 3 µM (±)-desmethoxyverapamil, was biphasic (Table II). If one calculates the kinetically derived $K_D$ values from the DMBODIPY-PAA rate constants, 15.56 and 0.71 nM, respectively, are obtained. Neither of these values is close to the equilibrium binding $K_D$ value.

The pharmacological profile of DMBODIPY-PAA-labeled Ca²⁺ channels is shown in Fig. 3, A and B. Interestingly, (+)-PN200-110 inhibited DMBODIPY-PAA binding with an $IC_{50}$ value of 19.2 ± 6.5 nM. The inhibition is stereospecific as (+)-PN200-110 is much less active ($IC_{50}$ value: 614 ± 31 nM). In contrast, (+)-PN200-110 stimulates the binding of (-)-[³H]desmethoxyverapamil to purified channels (14) as is illustrated in Fig. 3C. Taken together, DMBODIPY-PAA binds reversibly and with high affinity to purified L-type Ca²⁺ channels. The main differences with respect to other phenylalkylamines are an allosteric inhibition by the potent dihydropyridine Ca²⁺ antagonist (+)-PN200-110 and its increased affinity for purified Ca²⁺ channels in comparison with the commonly employed radioligands (-)-[³H]desmethoxyverapamil or [N-methyl-³H]LU49888.

### Purification of Rabbit Skeletal Muscle L-type Ca²⁺ Channels

**Fluorescent Phenylalkylamine and L-type Ca²⁺ Channels**

with the aid of DMBODIPY-PAA — In Fig. 4 we demonstrate that a prelabeling protocol with DMBODIPY-PAA can be employed to purify Ca²⁺ channels from skeletal muscle transverse tubule membranes. At 2 °C sufficient label remained bound to the phenylalkylamine site up to the sucrose gradient centrifugation step. Postlabeling of the dihydropyridine receptors with (+)-[³H]PN200-110 indicated that both binding sites co-purify.

**Phenylalkylamine Binding to Purified Ca²⁺ Channels Is Dependent on Ca²⁺—Phenylalkylamine binding to purified Ca²⁺ channels at 22 °C is inhibited by the chelator EDTA as shown in Fig. 5A. Maximal inhibition was 98% when (-)-[³H]desmethoxyverapamil labeled the channel and 84% when DMBODIPY-PAA was the ligand. Similar findings were obtained with EGTA (not shown). In contrast, phenylalkylamine binding to the membrane-bound Ca²⁺ channel was inhibited maximally by only 27% at the same temperature. We have measured the dependence of phenylalkylamine binding on free Ca²⁺ (employing DMBODIPY-PAA) for purified Ca²⁺ channels. The $K_D$ value for free Ca²⁺ for 100% restoration of binding was 58 nM (Fig. 5B). This value is in the range reported earlier for restoration of dihydropyridine binding (30.1–301 nM, depending on conditions, see Refs. 27 and 28). At Ca²⁺ concentrations >30 µM phenylalkylamine binding was inhibited with an $IC_{50}$ value ∼300 µM (not shown).

**Temperature and Time Dependence and Reversibility of the Chelator Effects**—We have investigated the temperature de-

### Table II

Rate constants for phenylalkylamine and dihydropyridine receptors

In this table rate constants obtained under different experimental conditions are summarized. In the first column we indicate the ligand with which the channel was labeled at equilibrium at 22 °C and the additions by which equilibrium was perturbed. For further details, including concentrations of ligands and channel proteins see "Materials and Methods" and the legend of Fig. 7. At equilibrium either EDTA (200 µM) or a nonspecific binding definition of the respective ligand was added to initiate either conversion or dissociation, respectively. In some experiments both conversion (by EDTA) and ligand dissociation (by blockade of the forward reaction) were initiated at equilibrium. These experiments are referred to in the text as “double-chase” experiments. Constants obtained without allosteric regulators are summarized in the second column (control conditions). Other channel preparations received a positive allosteric heterotropic regulator together with the ligand at the start of the binding experiment. The presence or absence of the regulator and its concentration is indicated in the third column. For experiments where the data could be only fitted by two exponential functions the percentage of sites contributing to the respective rate is given in parenthesis. In case where $n$, the number of experiments, is ≥3 the data are given ± S.E. If $n$ is 1 the asymptotic standard deviation of the nonlinear regression curve fit is presented. ND, not determined.

<table>
<thead>
<tr>
<th>Ligated/conditions</th>
<th>Control conditions</th>
<th>Allosteric regulator present</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>$k_{-1} = 1.48 ± 0.96$</td>
<td>$k_{-1} = 0.098 ± 0.11$</td>
</tr>
<tr>
<td>EDTA</td>
<td>$k_{+1} = 0.044 ± 0.023$</td>
<td>$k_{+1} = 0.009 ± 0.01$</td>
</tr>
<tr>
<td>3 µM (±)-D-888</td>
<td>$k_{-1} = 5.02 ± 0.62$</td>
<td>$k_{-1} = 0.055 ± 0.01$</td>
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<tr>
<td>(-)-[³H]Desmethoxyverapamil</td>
<td>$k_{+1} = 0.214 ± 0.12$</td>
<td>$k_{+1} = 2.38 ± 0.19$</td>
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<tr>
<td>EDTA</td>
<td>$k_{+1} = 1.37 ± 0.91$</td>
<td>$k_{+1} = 0.93 ± 0.08$</td>
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<tr>
<td>1 µM (-)-D888</td>
<td>$k_{+1} = 0.0412 ± 0.002$</td>
<td>$k_{+1} = 10.8 ± 0.25$</td>
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<tr>
<td>EDTA + 1 µM (-)-D888</td>
<td>$k_{+1} = 2.94 ± 1.86$</td>
<td>$k_{+1} = 0.055 ± 0.01$</td>
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<tr>
<td>(+)-[³H]PN200-110</td>
<td>$k_{-1} = 6.18 ± 0.89$</td>
<td>$k_{-1} = 0.055 ± 0.01$</td>
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<tr>
<td>EDTA</td>
<td>$k_{-1} = 0.569 ± 0.124$</td>
<td>$k_{-1} = 2.38 ± 0.19$</td>
</tr>
<tr>
<td>1 µM (+)-PN200-110</td>
<td>$k_{-1} = 0.024 ± 0.006$</td>
<td>$k_{-1} = 0.0164 ± 0.009$</td>
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<tr>
<td>(+)-[³H]nitrendipine</td>
<td>$k_{-1} = 0.0352 ± 0.003$</td>
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<td>1 µM (+)-PN200-110</td>
<td>$k_{-1} = 1.02 ± 0.14$</td>
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fluorescent phenylalkylamine and L-type Ca\textsuperscript{2+} Channels

**FIG. 3.** Pharmacological profile of purified L-type Ca\textsuperscript{2+} channels labeled by DMBO-DIPY-PAA. Purified rabbit skeletal muscle L-type Ca\textsuperscript{2+} channel preparations (0.018–0.0207 mg/ml of protein) were incubated with 3.62–5.37 nM DMBO-DIPY-PAA for 30 min at 22 °C in a final assay volume of 2 ml. Specifically bound ligand at equilibrium ranged from 0.91 to 1.03 nM. A, inhibition profile by different phenylalkylamines. The following IC\textsubscript{50} values and pseudo Hill coefficients (n\textsubscript{H}) were obtained by computer analysis: (-)-D888 (●): IC\textsubscript{50} 12.12 ± 0.34 nM, n\textsubscript{H} 1.03 ± 0.06; (+)-D888 (○): IC\textsubscript{50} 17.20 ± 0.12 nM, n\textsubscript{H} 1.03 ± 0.06; (-)-D600 (■): IC\textsubscript{50} 69.13 ± 0.89 nM, n\textsubscript{H} 0.56 ± 0.12; (+)-D600 (▲): IC\textsubscript{50} 65.46 ± 1.45, n\textsubscript{H} 0.69 ± 0.12; (-)-verapamil (▲): IC\textsubscript{50} 257.2 ± 8.0 nM, n\textsubscript{H} 0.89 ± 0.16 nM; (+)-verapamil (Δ): IC\textsubscript{50} 496 ± 12.4 nM, n\textsubscript{H} 1.04 ± 0.11; (-)-SU4888 (□): IC\textsubscript{50} 35.66 ± 2.3 nM, n\textsubscript{H} 1.05 ± 0.06; D619 (□): IC\textsubscript{50} 12670 ± 411 nM, n\textsubscript{H} = 1.18 ± 0.07. B, inhibition profile for non-phenylalkylamine L-type Ca\textsuperscript{2+} channel ligands. (+)-cis-diltiazem (●): IC\textsubscript{50} 21.40 ± 21 nM, n\textsubscript{H} 0.75 ± 0.08; (-)-cis-diltiazem (○): IC\textsubscript{50} 14,150 ± 345 nM, n\textsubscript{H} 1.38 ± 0.23; (-)-BM 20,1140 (□); IC\textsubscript{50} 311.1 ± 10.4 nM, n\textsubscript{H} 0.88 ± 0.06; (+)-BM 20,1150 (△): IC\textsubscript{50} 250 ± 9.4 nM, n\textsubscript{H} 0.98 ± 0.1. C, differential regulation of phenylalkylamine binding to purified L-type Ca\textsuperscript{2+} channels by (+)-PN200-110 (0): IC\textsubscript{50} 0.71 ± 0.04. 0.014 mg of protein/ml were incubated with 0.026 mg/ml of purified L-type Ca\textsuperscript{2+} channel protein for 30 min at 22 °C in a final assay volume of 2 ml. Specific binding was measured by fluorescence (open bars, picomoles/2000 pl). EC\textsubscript{50}, IC\textsubscript{50}, 100% restoration of control binding value was obtained in buffer A. EC\textsubscript{50}, IC\textsubscript{50}, 100% restoration of control binding value was obtained in buffer A. IC\textsubscript{50}, IC\textsubscript{50}, 100% restoration of control binding value was obtained in buffer A.

**FIG. 4.** Purification of rabbit skeletal muscle L-type Ca\textsuperscript{2+} channels after prelabeling with DMBO-DIPY-PAA. Shown are: phenylalkylamine receptor-bound DMBO-DIPY-PAA, measured by fluorescence (open bars, picomoles/2000 ml, scaled on the left y axes) and specific (+)-[3H]PN200-110 binding to the same fraction (filled diamonds, connected by dashed lines, picomoles/2000 ml, scaled on the right y axes) and the protein profiles (solid lines, micrograms/500 liters, scaled on left y axes). A, elution profile of a DMBO-DIPY-PAA labeled and dibromopyridine receptor postlabeled Ca\textsuperscript{2+} channel preparation from a wheat germ agglutinin-Sepharose column. Only the biospecific elution profile by 5% N-acetylglucosamine is shown. B, DMBO-DIPY-PAA fluorescence and dibromopyridine receptor binding (postlabeling) profile after separation of the fractions eluted above by sucrose gradient centrifugation.

dependence of the chelator effect and found that fewer channels were sensitive to the removal of divalent cations as temperature decreased (10 or 0 °C). At 0 °C and 240 min of incubation in 0.2 or 1 mM EDTA, the maximal inhibition was only 55% (not shown).

For simplicity we will refer to the different channel states with respect to phenylalkylamine binding as “CH\textsubscript{o},” “CH\textsubscript{1},” and “CH\textsubscript{2},” “CH\textsubscript{o},” CH\textsubscript{o} is a very low affinity binding state of the channel which can be achieved by removal of divalent cations. Under our incubation conditions, a fraction of the channels were apparently resistant to 0.2 or 1 mM EDTA (see Fig. 5A). This apparently resistant fraction could also be increased by adding 0.2 mM EDTA. The state “CH\textsubscript{2},” “CH\textsubscript{2},” as will be explained in more detail in the next section, this state results from the time chosen for incubation and the kinetics of the conversion process. The fraction of channels that could be converted under these conditions into CH\textsubscript{o} was termed “CH\textsubscript{o}.”

An important question is whether this chelator effect (CH\textsubscript{i} → CH\textsubscript{o}) simply reflects an irreversible denaturation process.

To answer this question we performed kinetic studies with a temperature-jump protocol (from 22 to 0 °C). In these studies we initiated the conversion (CH\textsubscript{i} → CH\textsubscript{o}) at 22 °C by adding 0.2 mM EDTA. At given time points channels were removed and exposed to either excess Ca\textsuperscript{2+} (0.05 mM) or kept in 0.2 mM EDTA or in 2 mM EDTA. All of the channels were subsequently exposed to the radioligand for 240 min at 0 °C and specific binding determined as described under “Materials and Methods.” The rationale behind this approach was as follows: if Ca\textsuperscript{2+} removal was accompanied by an irreversible

For reasons of clarity the rate constants for CH\textsubscript{i} → CH\textsubscript{o} are termed “conversion” constants.
inactivation, recovery would not be possible by the addition of Ca++. The data in Fig. 6 indicate that the conversion of the channels from CH4 to CH2 occurred in a biphase manner (see Table II). When Ca++ was added in excess above EDTA, phenylalkylamine binding completely recovered. The t1/2 of the recovery process was dependent on the time channels were kept at 22 °C. The data also indicate that, once the conversion CH4 → CH2 is initiated at 22 °C, lowering the temperature to 0 °C alone does not lead to recovery of phenylalkylamine binding. Instead, the conversion proceeds at 0 °C and is nearly complete after 240 min. Thus, at 0 °C phenylalkylamine binding cannot be independent from Ca++. The importance of temperature is also underlined by experiments in which 0.05 mM Ca++ was added but the channels immediately cooled to 0 °C and kept for 240 min at this temperature. The recovery (CH2 → CH4) occurred with an extremely slow rate compared with channels that were kept for different times at 22 °C and subsequently exposed to radioligand at 0 °C for 240 min.

Allosteric Regulation of the Conversion and Dissociation Kinetics—Dihydropyridines can inhibit the equilibrium binding of phenylalkylamines to membrane-bound L-type Ca++ channels (2-4, 52). In contrast, phenylalkylamine binding to purified L-type Ca++ channels is either stimulated or inhibited by dihydropyridines, depending on structure and orientation of the ester side chain (14). For example, the potent Ca++ antagonists (+)-PN200-110 and (R)-202-791 increased (−)-[3H]desmethoxyverapamil binding mainly by decreasing the equilibrium dissociation constant, whereas the agonist (S)-202-791 was inhibitory (14). Fig 3C showed that DMBODIPY-PA binding to purified Ca++ channels was inhibited by (+)-PN200-110, whereas (−)-[3H]desmethoxyverapamil binding was stimulated as reported previously (14). These allosteric effects are reciprocal for purified Ca++ channel preparations as reported above.

It was of interest to study the effects of allosteric regulators on the conversion of channels induced by Ca++ removal. In Fig. 7A we show an example where (+)-PN200-110 was added simultaneously with EDTA after DMBODIPY-PA reached equilibrium with the Ca++ channel. Clearly, the dissociation rate is increased and supports the evidence that (+)-PN200-110 acts as a negative heterotropic allosteric regulator for

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**Fig. 6. Temperature-jump experiments with purified Ca++ channels.** All filled symbols in this graph represent data points which were obtained after 240 min (additional) incubation at 0 °C and show the recovery of Ca++ currents at 0 °C (open circles) or 22 °C (open squares). Rate constants are summarized in Table II. At 60 min at 22 °C (not shown in the graph) the inhibition of (−)-[3H]desmethoxyverapamil binding by 200 μM EDTA reached 2% of control binding at equilibrium, which did not decay >10% over the entire time course shown. In a parallel experiment Ca++ channels (226 μg of protein/ml) were preincubated (without radioligand) for 2 min in a volume of 2 ml of buffer B at 22 °C in 200 μM EDTA. The reaction started at time 0 by addition of EDTA (to 200 μM) which contained (−)-[3H]desmethoxyverapamil (0.836 nM in the absence and presence of 3 μM (±)-desmethoxyverapamil) and 200 μM EDTA (■) or 200 μM EDTA plus 250 μM Ca++ (as CaCl2) (●) or buffer B only (▲). EDTA under the latter conditions was thereby decreased to 2 μM; at 0 °C and 240-min incubation there was no inhibition of radioligand binding by 2 μM EDTA (not shown). Data obtained from adding Ca++ in excess above EDTA, but leaving Ca++ channels at 0 °C (●) are connected by dashed arrows to the 2- or 20-min time point of the phenylalkylamine receptor conversion curve to indicate the percentage of control binding that was reached after jumping to 0 °C. These data points have been placed in the figure at arbitrarily chosen 22 °C time points, solely for reasons of space and clarity of presentation. Note that, when Ca++ was added in excess above EDTA and when channels were kept at 22 °C (●) (for the times shown in the graph) before cooling to 0 °C and keeping them for an additional 240 min at this temperature, control binding was reached with a t1/2 ≈0.5 min for the 2-min point and with a t1/2 > 2 min for the 20-min point. These data are connected by solid lines. Incubation of the Ca++ channel with EDTA in the absence of radioligand at 22 °C, cooling to 0 °C and simultaneously lowering EDTA to concentrations (2 μM) which do not inhibit phenylalkylamine labeling at 0 °C, yields data points (▲) that are indistinguishable from those obtained by having the radioligand at equilibrium with Ca++ channels first and initiating dissociation by adding EDTA at 22 °C, without a temperature-jump.

**Fig. 7. Dissociation and conversion kinetics of purified L-type Ca++ channels.** DMBODIPY-PA binding kinetics: A, purified Ca++ channels (0.029-0.041 mg of protein/ml) were labeled with 5.4-8.7 nM DMBODIPY-PA for 30 min at 22 °C. Specifically bound ligand at equilibrium was 1.15-2.01 nM. Dissociation was initiated by addition of 200 μM EDTA (●), simultaneous addition of 200 μM EDTA and 1 μM (+)-PN200-110 (○) or addition of 3 μM (±)-desmethoxyverapamil (▲). The following rate constants were obtained: αi, k−1, 2.34 ± 0.45 min−1; ki, 0.038 ± 0.006 min−1; n, k2, 2.38 ± 0.94 min−1, k3, 0.069 ± 0.011 min−1, ti, 4.90 ± 0.85 min−1, ki, 0.129 ± 0.006 min−1. The data could be better fitted by a two-site model than by a mono-exponential function. Dihydropyridine receptor kinetics: B, a purified Ca++ channel preparation (0.008-0.017 mg of protein/ml) was incubated with 0.493-0.646 nM (+)-[3H]PN200-110 in the absence (filled symbols = control) or presence of 1 μM (±)-desmethoxyverapamil (open symbols) for 30 min at 22 °C. Specific (+)-[3H]PN200-110 binding at equilibrium was 60.1-54.3 pm. Dissociation of bound radioligand was initiated by addition of 200 μM EDTA (●), 1 μM (+)-PN200-110 (▲), 200 μM EDTA and 1 μM (+)-PN200-110 (○), or 200 μM EDTA in the presence of 1 μM (±)-desmethoxyverapamil (●). The following rate constants were obtained by linear regression analysis of a logarithmic transformation of the data: αi, 0.0253 ± 0.003 min−1; k−1, 0.99; ki, 0.0297 = 0.002 min−1; n, 0.96; ti, 0.098 ± 0.011 min−1, r = 0.97; ti, 0.0152 ± 0.004 min−1, r = 0.99; k2, 3.035 ± 0.001 min−1, r = 0.97; t-, 0.97; k3, 0.129 ± 0.006 min−1. The following rate constants for (+)-[3H]desmethoxyverapamil binding: C, 0.617-1.22 nm (−)-[3H]desmethoxyverapamil were incubated with 0.008-0.017 mg of purified Ca++ channel protein/ml in the absence (filled symbols, control) or presence of 1 μM (+)-PN200-110 (open symbols) for 30 min at 22 °C. Equilibrium bound radioligand was 61.2-145 pm under control conditions and 111-245 pm in the presence of 1 μM (+)-PN200-110. Dissociation was initiated by addition of 200 μM EDTA (●), 3 μM (±)-desmethoxyverapamil (△), or 200 μM EDTA and 3 μM (±)-desmethoxyverapamil (●). The following rate constants were obtained by computer-fitting: αi, 0.97 ± 0.011 min−1, k−1, 0.0364 ± 0.007 min−1; k−2, 4.78 ± 1.41 min−1, k−3, 0.463 ± 0.11 min−1; n, 2.22 ± 0.96 min−1, ti, 0.076 ± 0.01 min−1.
DMBODIPY-PAA-labeled channels. When the dihydropyridine receptors of the Ca\textsuperscript{2+} channels were incubated with a saturating concentration (1 \( \mu \text{M} \)) of (+)-PN200-110 (-\textsuperscript{[3H]} desmethoxyverapamil binding was significantly less sensitive to Ca\textsuperscript{2+} removal, whereas DMBODIPY-PAA labeling remained essentially unchanged with respect to chelator sensitivity (Fig. 5A). These observations prompted us to investigate in more detail the time dependence of the conversion to the low-affinity phenylalkylamine state (CH\textsubscript{A}) and the influence of heterotropic allosteric regulators (i.e. the dihydropyridine (+)-PN200-110) on this process. In contrast to the kinetic experiment shown in Fig. 7A, heterotropic allosteric regulators and the ligand were equilibrated with the channels. The equilibrium was then perturbed by addition of chelator, unlabelled phenylalkylamine, or a combination of the two. The data are summarized in Table II and examples are shown in Fig. 7, B and C. As a comparison, data obtained with the dihydropyridine radioligands (+)-\textsuperscript{[3H]}PN200-110 and (±)-\textsuperscript{[3H]}nitrendipine are included.

In the absence of allosteric regulators we found that the two conversion rate constants \((k_{1}, k_{-1})\) were indistinguishable for both phenylalkylamines. This was surprising, taking into account the different equilibrium binding and allosteric properties. Dissociation, initiated by an excess of unlabeled ligand, is significantly faster for both phenylalkylamines than conversion induced by Ca\textsuperscript{2+} removal. If these processes can occur independently (and not consecutively), the faster rate (i.e. dissociation) must dominate the overall complex decay in double-chase experiments with EDTA and excess drug. This was indeed the case, as shown in Table II, where even additivity is suggested.

Next we investigated the effects of a saturating concentration of (+)-PN200-110 on the conversion rates (CH\textsubscript{A} \( \rightarrow \) CH\textsubscript{B}) when the channel was labeled by (-\textsuperscript{[3H]} desmethoxyverapamil. These rates were decreased by (+)-PN200-110. (-)-\textsuperscript{[3H]} desmethoxyverapamil dissociation, on the other hand, was no longer biphasic in the presence of (+)-PN200-110. However, in double-chase experiments biphasic dissociation was observed despite the presence of (+)-PN200-110, and both rate constants were considerably lower.

In striking contrast to phenylalkylamine labeled Ca\textsuperscript{2+} channels, conversion measured with dihydropyridines was always strictly monophasic. The conversion rates are of similar magnitude as the slower rate constants \((k_{1}, k_{-1})\) for the two phenylalkylamines. The conversion rate was decreased from 0.0237 min\textsuperscript{-1} to 0.0164 min\textsuperscript{-1} for the (+)-\textsuperscript{[3H]}PN200-110 labeled channel when the positive allosteric regulator (-)-desmethoxyverapamil was present.

The measured rate constants allow us to predict the times required to reach, for example, \( \approx 10\% \) of the original (control) binding after addition of 0.2 mM EDTA. With the rate constants obtained for the phenylalkylamines (in the absence of dihydropyridine), >90\% of the sites are predicted to be in the CH\textsubscript{A} state after \( \approx 21 \text{ min} \). In the presence of (+)-PN200-110 this conversion is considerably slower when (-)-\textsuperscript{[3H]}desmethoxyverapamil labels the channel: \( \approx 11 \text{ h} \) at 22 \( ^\circ \text{C} \) are required to reach 10\% of the control binding. These rate constants explain that the apparently chelator-insensitive channel fraction (CH\textsubscript{B}) is solely a consequence of the kinetics of the conversion process and our limited incubation time. The half-life of the channel drug binding sites is \( \approx 2.5 \text{ h} \) at 25 \( ^\circ \text{C} \) (43), and four incubation times were chosen to reach equilibrium for drugs without having to correct for channel denaturation. Although we have not measured the kinetics of the conversion at 0 \( ^\circ \text{C} \), it is very likely that the greater proportion of the apparently chelator-insensitive channel fraction at lower temperatures has a similar kinetic basis.

These kinetic studies suggest a heterogeneity of Ca\textsuperscript{2+} channel phenylalkylamine receptors revealed by divalent cation removal. In contrast, a homogeneous population of dihydropyridine receptors exists as shown by a strictly monoeponential conversion. This monophasic process prevailed whether or not (-)-desmethoxyverapamil (which stimulates (+)-\textsuperscript{[3H]}PN200-110 binding) occupied the phenylalkylamine site. We conclude that the allosteric interaction between the phenylalkylamine and dihydropyridine sites can be observed under two different kinetic conditions: namely conversion of the channels into a very low affinity binding state (induced by removal of divalent cations with EDTA) or by blockade of the ligand association reaction.

**DISCUSSION**

*Properties of the Fluorescent Phenylalkylamine*—This study introduces the first fluorescent phenylalkylamine (DMBODIPY-PAA) for Ca\textsuperscript{2+} channel research and presents a detailed analysis of L-type Ca\textsuperscript{2+} channel drug receptor kinetics. DMBODIPY-PAA can be employed to characterize purified L-type Ca\textsuperscript{2+} channels by measuring channel-bound fluorescence after removal of unbound fluorescent label by charcoal adsorption and cooling to 2 \( ^\circ \text{C} \). Association and dissociation kinetics, equilibrium saturation experiments, and drug interaction studies can be performed with the aid of a spectrofluorimeter. The purification of the L-type Ca\textsuperscript{2+} channel with a prelabeling protocol could also be demonstrated. Fluorescent ligands have been previously developed for different receptor types, e.g. for glycine (40) or \( \beta \)-adrenergic receptors (48). With respect to voltage-regulated or ligand-gated ion channels, fluorescent probes exist for (nicotinic) acetylcholine receptors (53), voltage-dependent Na\textsuperscript{+} channels (54, 55), and \( \omega \)-conotoxin GVIA-sensitive (N-type) Ca\textsuperscript{2+} channels (56). In general these fluorescent ligands have been useful to study the distribution of their binding sites at the subcellular level or their lateral diffusion (see e.g. Refs. 40 and 56) or to estimate distances between different toxin binding sites on, e.g. Na\textsuperscript{+}channels (57). In no case, however, have these ligands gained general acceptance to replace radioactive ligands. DMBODIPY-PAA, as shown above, has this potential especially if other methods, including fluorescence anisotropy, are applicable. In competition studies with membrane-bound and purified Ca\textsuperscript{2+} channels from skeletal muscle transverse tubule membranes the characteristic loss of phenylalkylamine receptor affinity, which occurs upon Ca\textsuperscript{2+} channel purification, was not exhibited by DMBODIPY-PAA. Likewise, the IC\textsubscript{50} value for competition with the standard radioligand (±)-\textsuperscript{[3H]}desmethoxyverapamil decreased, whereas (±)-desmethoxyverapamil and other phenylalkylamines displayed an increase when membrane-bound and purified Ca\textsuperscript{2+} channels were compared. The loss of affinity for (±)-\textsuperscript{[3H]}desmethoxyverapamil upon purification does not reflect an irreversible denaturation. Others have reconstituted purified L-type Ca\textsuperscript{2+} channels from rabbit skeletal muscle and observed identical equilibrium binding constants for phenylalkylamines as in membranes (37). Addition of (+)-PN200-110 to purified channels leads to increased affinity for (±)-desmethoxyverapamil but lowers the affinity for DMBODIPY-PAA and accelerates its dissociation. We offer the following hypothesis to explain these phenomena: when Ca\textsuperscript{2+} channels are purified, the phenylalkylamine binding site undergoes a conformational change that leads to a \( \approx 10\)-fold loss of affinity for (±)-desmethoxyverapamil, consistent with the loss of one weak, perhaps a van der Waal, bond (\( \approx 1 \text{ kcal/mol} \)). This bond may not be formed by DMBODIPY-PAA. Instead, optimal contact with the large
bulky side chain of DMBODIPY-PAA could be favored, which may not be possible in the membrane-bound state, perhaps due to the steric hindrance. When the dihydropyridine (+)-PN200-110 binds to the purified Ca\(^{2+}\) channel it can reverse, at least in part, these changes, mimicking the membrane-bound state. An intriguing finding is that these interactions are reciprocal: (+)-desmethoxyverapamil favored the binding of (+)-PN200-110, whereas DMBODIPY-PAA inhibited it.

We believe that this is strong evidence for tight reciprocal coupling of the dihydropyridine and phenylalkylamine binding domain in the folded structure of the \(\alpha_1\)-subunit.

Since DMBODIPY-PAA fluorescence was enhanced in the presence of 0.1% (w/v) digitonin (the detergent in which the channel is solubilized and isolated), direct labeling of channels was facilitated. Titration of a fixed DMBODIPY-PAA concentration with purified Ca\(^{2+}\) channels revealed that 50% of our ligand binds with a \(K_d\) value which is nearly one order of magnitude lower than that of available radioligands from this class of compounds. The signal-to-noise ratio was good, but an optically pure enantiomer may even increase this ratio. Heterogeneity of the phenylalkylamine sites in equilibrium saturation analysis (either by titration of the ligand with receptors or by DMBODIPY-PAA saturation of Ca\(^{2+}\) channels) was not apparent, and the \(K_d\) values for both types of analysis were in very good agreement. They also agreed with the (−)-[\(^3\)H]desmethoxyverapamil competition IC\(_{50}\) values when 50% of the added DMBODIPY-PAA is regarded as receptor-inactive. However, apparent heterogeneity of the binding sites is suggested by the biphasic dissociation behavior of DMBODIPY-PAA. Neither of the two rate constants (\(k_1\), \(k_2\)) yields the equilibrium saturation \(K_d\) when the measured \(k_1\), is employed for calculation. Possibilities, which we have not yet vigorously excluded, are heterogeneity with respect to the association reaction or more complex reaction schemes. Similar observations (biphasic dissociation) were made for the radioligand (−)-[\(^3\)H]desmethoxyverapamil, although a filtration technique for separation of bound and free label was employed. Interestingly (−)-[\(^3\)H]desmethoxyverapamil dissociated in a strictly mono-exponential fashion when the dihydropyridine (+)-PN200-110 was bound to the channel.

Ca\(^{2+}\) Binding Sites of the Ca\(^{2+}\) Channel—At 22 °C the majority of the channels could be converted within 30 min into a divalent cation-depleted state by addition of chelators. These channels had lost the ability to bind phenylalkylamines or dihydropyridines with high affinity. For the phenylalkylamines, the time-dependent conversion was biphasic, and the two rate constants were identical for both the radioligand and fluorescent probe. These constants are lower than those measured for dissociation of the two phenylalkylamine probes. Evidence obtained in double-chase experiments indicated that dissociation induced by a blockade of the forward reaction of the drug and drug binding site conversion, induced by chelators, occurs independently. In contrast to the phenylalkylamine receptor, the conversion was governed by mono-exponential kinetics when the dihydropyridine receptor was probed with (−)-[\(^3\)H]PN200-110 or (±)-[\(^3\)H]nitrendipine. Remarkably, the slower rate constants (0.041 or 0.041 min\(^{-1}\), see Table II) for the chelator-induced conversion of the drug binding sites, measured with the phenylalkylamines, are in the same range as the constants obtained with the two dihydropyridines. Similar to the phenylalkylamines, the channel conversion rates measured with radiolabeled dihydropyridines appear to be independent from the dissociation rates of the ligands. These observations suggest that the underlying mechanism is the same for ≈60% of the labeled phenylalkylamine sites and for all of the labeled dihydropyridine sites. In addition to heterogeneity of phenylalkylamine sites other possibilities exist, for example, negative cooperativity.

As reported elsewhere, dihydropyridine binding to purified L-type Ca\(^{2+}\) channels is dependent on free Ca\(^{2+}\) (27, 28). In our experiments we can demonstrate that the conversion into the very low affinity phenylalkylamine binding state is a completely reversible process with a \(K_{ds}\) value for free Ca\(^{2+}\) of 58 nM. The back reaction, induced by Ca\(^{2+}\), is extremely temperature-dependent and it requires <5 min for full recovery at 22 °C but >4 h at 0 °C for ≈20% recovery of control binding. In contrast to the recovery, the temperature dependence of the conversion time course has not yet been examined by us. Experiments at 0 °C with EDTA (at concentrations that induce >90% of the decay at 22 °C) suggest that this process could be as slow as the recovery at 0 °C. We were initially puzzled by the observation that stimulators of phenylalkylamine binding [(±)-PN200-110 for (−)-[\(^3\)H]desmethoxyverapamil] and temperature decrease enhanced the fraction of channels that were apparently resistant to Ca\(^{2+}\) removal. Kinetic studies, however, revealed that this resistance resulted from an incubation time which did not allow for complete conversion into the divalent cation-depleted state. The possibility that this could be due to Ca\(^{2+}\)-independent binding at 0 °C can be excluded by our temperature jump studies.

The ion selectivity of the divalent cation site, which is coupled in a positive manner to the drug binding sites, has so far been only studied in more detail for membrane-bound dihydropyridine receptors (see Refs. 2 and 4 for reviews). When this metal binding site is formed by charged amino acids, it is conceivable that the most abundant cation (Tris\(^{+}\)) replaced Ca\(^{2+}\) under our experimental conditions. It would be therefore of interest to study other organic and metal cations which either block or promote excitation-contraction coupling (34).

Ca\(^{2+}\) Dissociation or Isomerization of the Channel—Here we discuss problems that are encountered in the interpretation of our data and outline areas of future research. Consider a simple scheme, CH\(_3\) → CH\(_4\), where CH\(_3\) has Ca\(^{2+}\) bound and binds phenylalkylamines or dihydropyridines with high affinity, whereas CH\(_4\), depleted from Ca\(^{2+}\), does not bind or binds with very low affinity. It is very likely that CH\(_4\), once formed, dissociates the drug very rapidly. In this simple scheme conversion constants reflect Ca\(^{2+}\) dissociation rates and must therefore be independent from the radioligand employed. Rate constants of similar magnitude (0.024–0.044 min\(^{-1}\)) were indeed observed with the two dihydropyridines and with the two phenylalkylamines. However this statement holds for ≈60% of the phenylalkylamine sites only, which complicates the interpretation. Neglecting this complication we are confronted with the following problem: if the association of Ca\(^{2+}\) is purely diffusion-controlled (\(k_{on} \approx 10^8\) M\(^{-1}\) s\(^{-1}\)), and \(K_{ds}\) values for restoration of high affinity drug binding are taken as estimates of the Ca\(^{2+}\) affinity of the channel (\(K_{ds}\) around 100 nM (27, 28)), the expected dissociation rate constants are orders of magnitude higher than the rates that we actually measured. This paradox cannot be resolved unless either very high affinity for Ca\(^{2+}\) is postulated or constraints for the Ca\(^{2+}\) association are included. On the other hand, if a Ca\(^{2+}\) free intermediate (CH\(_{3}\)) is rapidly formed, which still binds the drug and the reaction proceeds as CH\(_3\) → CH\(_{4}\) → CH\(_{3}\), a slower isomerization rate (CH\(_{3}\) → CH\(_{3}\)) would dictate the overall rate. With such a mechanism our conversion constants do not reflect Ca\(^{2+}\) dissociation rate constants. The strong temperature dependence of the conversion induced by Ca\(^{2+}\) removal and of recovery after Ca\(^{2+}\) addition suggests confor-
Ca\(^{2+}\) affinity, but they cannot bind drugs. Inactivated states have high affinity for drugs but almost no affinity for Ca\(^{2+}\). These models help to explain that Ca\(^{2+}\) prevents inactivation, that phenylalkylamines stabilize the voltage sensor in a paralyzed inactivated state, and that Ca\(^{2+}\) (applied from the myoplasmic side) can antagonize the blocking effect of the phenylalkylamines. The state diagrams do not fully account for the effects of the dihydropyridines which are much more potent on ion permeation than on voltage sensor function (34) and make no assumptions on the number or orientation of the Ca\(^{2+}\) binding sites. Functional experiments argue for an extracellular orientation of the metal ion binding site which is the priming site and an intracellular Ca\(^{2+}\) site which is coupled to the phenylalkylamine binding domain (34). Our results are in accord with these functional data (Fig. 8). In this working model dihydropyridine and phenylalkylamine binding domains share a common high affinity Ca\(^{2+}\) binding site (Ca\(^{2+}\) site I). Ca\(^{2+}\) in this site is necessary for the stabilization of high-affinity drug binding domains. Its ion selectivity (2, 4, 28) is similar to that of the priming site (34, 35). Another Ca\(^{2+}\) binding site (Ca\(^{2+}\) site II) is coupled in a negative fashion to the phenylalkylamine binding site. We are tempted to speculate that Ca\(^{2+}\) site I is the priming site of the voltage sensor, oriented to the extracellular face of the Ca\(^{2+}\) channel, whereas Ca\(^{2+}\) site II is oriented to the cytosol.

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