Synthesis of Diazipine and [^{3}H]Diazipine: Novel Dihydropyridines as Photoaffinity Probes of Calcium Channels

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Diazipine and [^{3}H]diazipine were synthesized as new 1,4-dihydropyridine photoaffinity ligands containing a phenyldiazirine group. After simple high performance liquid chromatography separation, both compounds were purified in good overall yields. [^{3}H]Diazipine (21.2 Ci/mmol) was synthesized in two steps from commercially available [^{3}H]ethanolamine. Diazipine competitively inhibited [^{3}H]PN200-110 binding to the calcium channel of cardiac membranes with high affinity.

Keywords [^{3}H]diazipine; 1,4-dihydropyridine; calcium channel; photoaffinity ligand

Calcium channels are widely distributed in membrane, and are important for electrical excitability, excitation–contraction coupling, excitation–secretion coupling, and other cellular functions. A class of compounds known as 1,4-dihydropyridines (DHPs) functions either to block or activate voltage-regulated calcium channels in a variety of tissues and is, therefore, of considerable interest for both therapeutic and experimental purposes. A great number of DHPs have been developed and synthesized. DHPs are one of the most useful ligands to identify the L-type calcium channels, since they have high affinity even in broken cell preparations. DHPs bind to the alpha 1 subunit of the L-type channels.[1]

Photoaffinity labeling techniques using reagents with ligand structures have versatile roles to select and identify receptor molecules of the ligands in crude preparations, and even to locate or identify the binding sites of the ligands on the receptor molecules.[2] Azidopine is a currently employed photoreactive DHP with a nitrene precursor.[3] However, photoincorporated azidopine does not seem to be sufficiently chemically stable, and in fact, it was reported to be released to a considerable extent by reduction with dithiothreitol.[4] Such lability may hamper experiments to identify the labeled sites, for example. We have previously reported that phenyldiazirines are useful precursors to generate highly reactive carbenes which afford quite stable photoproducts.[5-7] As a extension of this line of work we report here the synthesis of novel photoactivatable dihydropyridines containing a phenyldiazirine.

A new dihydropyridine, 2-[4-(1-azi-2,2,2-trifluoroethyl)-benzoylamino]ethyl ethyl 2,6-dimethyl-4-(2-trifluoromethyl)phenyl-1,4-dihydropyridine-3,5-dicarboxylate, termed diazipine, has been synthesized as shown in Chart 1. The precursor dihydropyridine **1** is a common compound for azidopine synthesis. However, no synthetic details of **1** have been reported in the literature[8] and we prepared it by means of a conventional Hantzsch reaction[9] as described in the experimental section. Coupling of **1** with a phenyldiazirine **2** gave diazipine in good yield (80%) after high performance liquid chromatography (HPLC) purification.

In the binding experiments, diazipine competitively inhibited the [^{3}H](+)PN200-110 binding to calcium channels in cardiac membrane with an IC_{50} value of 3.81 nM (Fig. 1) or a K_i value of 1.48 nM. The binding affinity to the channel preparation was slightly lower than that of (−)azidopine (K_i = 1.05 nM), the active enantiomer of the currently used photoaffinity ligand, or of (±)PN200-110 (K_i = 0.96 nM), a typical DHP ligand, but the differences are only 1.5 times at most. Diazipine can be included in the

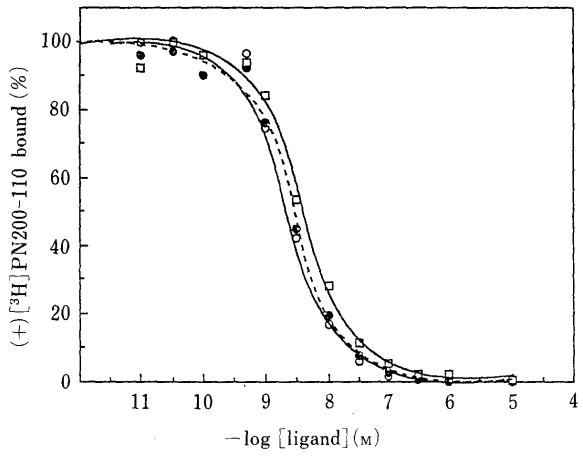
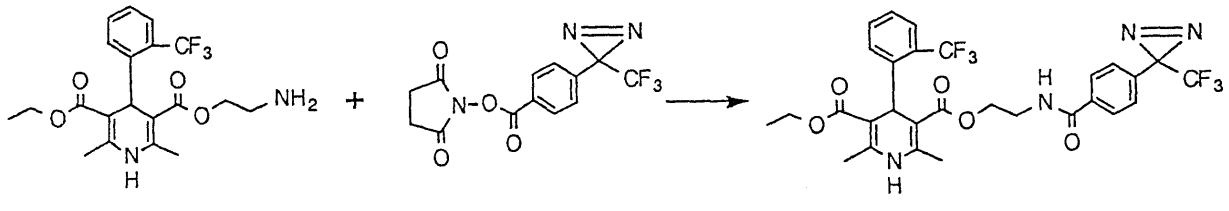


Fig. 1. Inhibition of [^{3}H](+)PN200-110 Binding by Dihydropyridines

□, diazipine; ●, (−)azidopine; ○, PN200-110. The experiment was performed by the incubation of various concentrations of each dihydropyridine with 200 μg/ml of porcine heart sarcolemma membranes and 0.17 nM [^{3}H](+)PN200-110 at 25 °C for 40 min.



1 2 diazipine

Chart 1. Synthetic Scheme for Diazipine

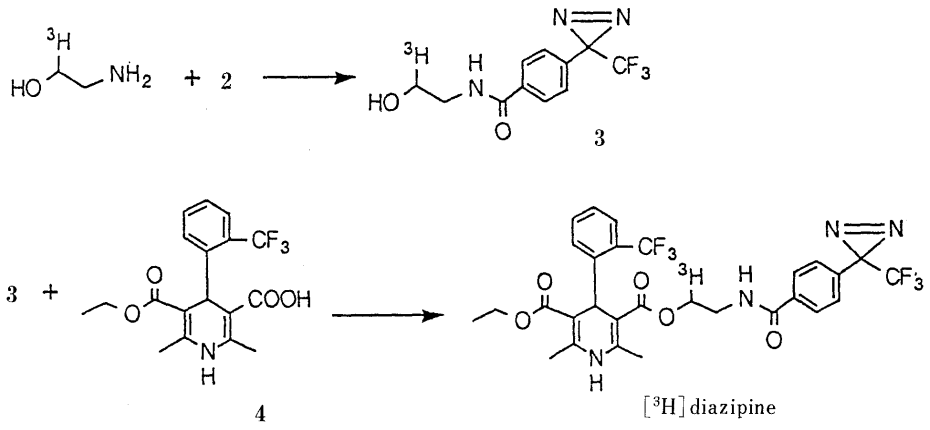


Chart 2. Synthetic Scheme for [^{3}H]Diazipine

series of DHP ligands for L-type calcium channels.

In the synthesis of [^{3}H]diazipine we used commercially available [1-^{3}H]ethan-1-ol-2-amine hydrochloride as a radioactive starting material and employed a different route than that used for the unlabeled diazipine, because as few steps as possible should be used in the synthesis of radioactive compounds. A two-step synthesis after introduction of [^{3}H]ethan-1-ol-2-amine was achieved, as shown in Chart 2. In order to optimize the radiosynthesis, all unlabeled reagents were used in 5- to 22-fold excess over the radioactive material. In the coupling of **3** with **4**, only the present method which is a modification of the described procedure[10] for this synthesis, gave a successful result. Other usual coupling methods which use acid chloride, dicyclohexylcarbodiimide, or carbonyldiimidazole, were unsuccessful. The overall yield of 40% after two reaction steps with subsequent HPLC purifications is satisfactory from a practical point of view. The specific radioactivity of 21.2 Ci/mmol is also good enough for studies on calcium channel preparations.

The above considerations, together with the result that diazipine has similarly high affinity to azidopine and PN200-110, suggest that diazipine, a novel dihydropyridine with phenyldiazirine group, will be useful for photoaffinity labeling of L-type calcium channels. An application study is under way.

Experimental

Instruments used in this study were as follows: MS, JEOL JMS-3000; proton nuclear magnetic resonance (^{1}H-NMR), JEOL JNX-FX 100, ultraviolet (UV), Hitachi UV-330, and liquid scintillation counter, Packard Tri-Carb 460C. HPLC was performed with a Waters HPLC system with two pumps (model 510) and an automated gradient controller. [^{3}H](+)PN200-110 was purchased from Amersham. (−)Azidopine was a kind gift from Prof. H. Glossmann and (±)PN200-110 was supplied by Sandoz.

Preparation of 1 2-(*N-tert*-Butyloxycarbonylamino)ethyl acetoacetate (2.45 g, 10 mmol), ethyl 3-aminocrotonate[11] (1.55 g, 12 mmol), and α,α,α-trifluoro-*o*-tolualdehyde (2.09 g, 12 mmol) were dissolved in ethanol (10 ml) and the solution was refluxed for 16 h. The solvent was evaporated off *in vacuo*, and the residue was purified by a silica-gel chromatography with benzene–ethyl acetate (2:1) as the solvent. A yellow oily material (2.59 g, 51% yield) was obtained. HRMS *m/z* Calcd for $C_{25}H_{31}F_3N_2O_6$: 512.21353. Found: 512.21062. The oil (2.56 g, 5 mmol) was dissolved in formic acid (19 ml) and the solution allowed to stand for 4 h at room temperature. After addition of water (25 ml), the aqueous solution was washed with chloroform three times. The aqueous layer was titrated to pH 11 with sodium hydroxide solution in an ice-bath followed by extraction with chloroform three times. The chloroform layer was dried over sodium sulfate, then the solvent was evaporated off *in vacuo*, and the residue was passed through an aluminum oxide column with chloroform–methanol (10:1) as the solvent to remove materials retained on top of the gel. The eluted fractions were evaporated to dryness *in vacuo* and the residue was recrystallized from benzene and *n*-hexane to give **1** as colorless prisms, 1.2 g (50% yield). mp 149.5—152.5°C, MS *m/z*: 412 (M^+). *Anal.* Calcd for $C_{20}H_{23}F_3N_2O_4$: C, 58.25; H, 5.62; N, 6.79. Found: C, 58.12; H, 5.62; N, 6.78. ^{1}H-NMR ($CDCl_3$) δ_{ppm}: 1.17 (t, 3H, J=7 Hz, C$\underline{H}_3$CH$_2$–), 1.47 (br, 2H, N$\underline{H}_2$CH$_2$–), 2.30, 2.32 (s, 6H, 2,6-dimethyl), 2.85 (t, 2H, J=5 Hz, –CH$_2$C$\underline{H}_2$NH$_2$), 3.9—4.3 (m, 4H, –COOC$\underline{H}_2$– × 2), 5.58 (s, 1H, 4-position of the dihydropyridine) 5.71 (s, 1H, –NH– at the 1-position of the dihydropyridine), 7.2—7.6 (m, 4H, $\underline{H}$-Ph × 4). UV λ_{max}^{EtOH} (ε): 355 (6100), 237 (18300).

Synthesis of Diazipine 4-(1-Azi-2,2,2-trifluoromethyl)benzoic acid *N*-hydroxysuccinimide ester (**2**)[12] (5 mg, 15 μmol) was dissolved in methylene dichloride (1 ml). Into this solution, 2-aminoethyl ethyl 2,6-dimethyl-4-(2-trifluoromethyl)phenyl-1,4-dihydropyridine-3,5-dicarboxylate **1**[8] (6.6 mg, 16 μmol) in methylene dichloride (0.2 ml) was added, and the mixture was stirred overnight at room temperature. The solvent was evaporated off *in vacuo*. The residue was dissolved in acetonitrile (0.2 ml) and 10 μl aliquots were separated by HPLC on a C18 column (Toso TSK 80TM, 4.6 × 250 mm) using CH_3OH–H_2O (4:1) as the solvent (flow rate: 0.5 ml/min). Eluates were monitored by using a UV detector at 240 nm and the peak at 15 min was collected as 2-[4-(1-azi-2,2,2-trifluoroethyl)benzoylamino]ethyl ethyl 2,6-dimethyl-4-(2-trifluoromethyl)-phenyl-1,4-dihydropyridine-3,5-dicarboxylate (termed diazipine). The overall yield of diazipine was 80%. *Anal.* Calcd for $C_{29}H_{26}F_6N_4O_5$: C, 55.77; H, 4.20; N, 8.97. Found: C, 55.58; H, 4.39; N, 8.75. MS *m/z*: 596 [$M^+ - N_2$]. UV λ_{max}^{EtOH} (ε): 356 (6330), 236 (32200).

Synthesis of [^{3}H]Diazipine Aqueous [1-^{3}H]ethan-1-ol-2-amine hydrochloride (223 nmol, 22.4 Ci/mmol from Amersham), was evaporated to dryness with a Speed-Vac (Savant) and the residue was dissolved in water (96 μl). To this, a 40 mM triethylamine solution in tetrahydrofuran (THF) (12 μl, 480 nmol), THF (190 μl), and finally a 50 mM solution of **2** in THF (22 μl, 1.1 μmol) were added and the whole was stirred for 12 h at room temperature. The reaction mixture was subjected to HPLC purification on a C18 column (Chemcosorb 7C18, 4.6 × 250 mm) with CH_3CN–H_2O (2:3) as the solvent (flow rate: 0.8 ml/min). The peak fractions eluted at 12 min was collected as the [^{3}H]ethanolamide derivative **3**. The fraction was evaporated to dryness and the residue was dried over P_2O_5 in a desiccator overnight *in vacuo*. The dry ethanolamide **3** (0.2 μmol) was dissolved in dry methylene dichloride (40 μl) and then 110 mM *N,N*-dimethylaminopyridine (40 μl, 4.4 μmol) was added to make 'solution A'. Separately, a 50 mM solution of 2,4-dinitrofluorobenzene (40 μl, 2 μmol) in dry methylene dichloride and a 50 mM solution of 2,6-dimethyl-4-(2-trifluoromethyl)phenyl-1,4-dihydropyridine-3,5-dicarboxylic acid monoethyl ester[13] (**4**) in dry methylene dichloride (40 μl, 2 μmol) was mixed to make 'solution B'. Solution B was added to solution A and the mixture was stirred for 12 h at room temperature. After evaporation of the solvent, the resultant residue was dissolved in acetonitrile (0.1 ml) and 5 μl aliquots were separated by HPLC in the same manner as described above for the unlabeled compound. A radiactive peak eluted at 15 min was collected. For further purification, pooled fractions were subjected to rechromatography under identical conditions. The overall yield of the purified [^{3}H]diazipine was 40% and its specific radioactivity was determined as

21.2 Ci/mmol, by measuring the radioactivity and absorption spectrum.

Binding Experiment Porcine heart sarcolemma membranes were prepared as described in the literature.[14] To the sarcolemma membranes (50 μg of protein) in 50 mM Tris–HCl (pH 7.4) containing 2 mM $CaCl_2$ 'binding buffer', either of diazipine, (−)azidopine, or PN200-110 (0.01 nM—10 μM) and then [^{3}H](+)PN200-110 (0.17 nM) were added, and the mixture (final volume: 250 μl) was incubated at 25 °C for 40 min. The incubation mixture was rapidly filtered through a Whatman GF/C filter on a filtration manifold (Brandel Cell Harvester M-24R) under reduced pressure, followed by rapid washing 5 times with cold binding buffer. Each of the filter disks was mixed with a scintillation cocktail and counted. Duplicate runs were performed for each data point.

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