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Diazipine, a novel photoaffinity probe for dihydropyridine receptors of calcium channels

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A new 1,4-dihydropyridine photoaffinity ligand, [^{3}H]diazipine, has been assessed by binding and photolabeling, and compared with a currently used [^{3}H]azidopine. [^{3}H]Diazipine reversibly binds to skeletal muscle Ca^{2+} channels with a similar affinity to [^{3}H]azidopine, but [^{3}H]diazipine labels the channel two times more efficiently and no release of the incorporated amount is observed after dithiothreitol treatment.

[^{3}H]Diazipine; Ca^{2+} channel; Photoaffinity label; Skeletal muscle

1. INTRODUCTION

Voltage regulated L-type calcium channels are widely distributed in many excitable cells of tissues such as skeletal, cardiac, and smooth muscle or brain. These channels have high affinity binding sites for a variety of drugs, e.g. 1,4-dihydropyridines, phenylalkylamines, benzothiazepines, and diphenylbutylpiperidines etc. (see [1] for review). The 1,4-dihydropyridines (DHP) are among the most useful ligands to identify the L-type calcium channels even in broken cell preparations. DHP binds to the α-1 subunit of the L-type channels [2]. Since the primary structures of α-1 subunits from various tissues (skeletal, cardiac, and smooth muscle) are known [3–5], it is a major goal to identify the DHP binding site(s) within the primary amino acid sequences.

Photoaffinity labeling plays an essential role to achieve this goal. Previously we have reported several reagents [6,7] and toxin derivatives for specifically photolabeling the sodium channels [8–10]. Recently we have synthesized diazipine (Fig. 1), a novel photoreactive derivative of DHP possessing a phenyldiazirine as a carbene precursor [11]. In this paper we describe the properties of diazipine, in terms of binding and photolabeling of the α-1 subunit in purified preparation of calcium channels from rabbit skeletal muscles. By comparison with azidopine, the currently used photoreactive DHP [12] characteristics of diazipine in binding affinity, photoincorporation efficiency, and photoproduct stability will also be described.

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2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]Diazipine (21.2 Ci/mmol) and unlabeled diazipine were prepared as described in [11]. [^{3}H]Azidopine (53.0 Ci/mmol) and [^{3}H](+)PN200-110 were purchased from Amersham and NEN, respectively. Unlabeled (±)PN200-110 was provided from Sandoz (Switzerland).

2.2. *Preparation of the WGA-Sepharose purified calcium channels*

Digitonin extracts of purified t-tubule fractions from rabbit skeletal muscles were purified by a WGA-Sepharose chromatography as described in the literature [2]. The *N*-acetylglucosamine eluates (specific binding activity of [^{3}H](+)PN200-110: 530–800 pmol/mg of protein) were used without further purification.

2.3. *Binding experiments*

Into the WGA-purified calcium channels (4 μg of protein) in 50 mM Tris-Cl (pH 7.4) containing 2 mM $CaCl_2$, 100 μM *cis*-diltiazem, and 0.1% digitonin 'binding buffer', [^{3}H]diazipine was added in various concentrations (1–80 nM) and final volumes were 500 μl. The mixture was incubated at 25°C for 1 h in the dark. Two 100 μl aliquots of the incubates were loaded on a Sephadex G-50 (fine) column (2 ml) which was pre-equilibrated with the binding buffer and gel-filtrated by centrifugation as described [13]. Radioactivity of the filtrate was measured by scintillation counting. Binding in the presence of 10 μM (±)PN200-110 was also carried out to measure non-specific binding. Duplicate runs were performed for each data point. For competitive binding experiments, unlabeled ligand (0.1 nM–10 μM) of either diazipine or (±)PN200-110 was incubated with 3.4 nM [^{3}H](+)PN200-110 and the purified calcium channel (1.9 μg of protein). Subsequent gel-filtration was carried out similarly.

2.4. *Photoaffinity labeling*

The WGA-purified calcium channel (30 μg/ml, 23 nM of DHP sites) was incubated with 11 nM of [^{3}H]diazipine or [^{3}H]azidopine in the binding buffer for 1 h at 25°C in the dark. An aliquot of the mixture was incubated in the presence of 10 μM (±)PN200-110 to determine nonspecific photolabeling. The incubation mixtures were transferred into plastic Petri dishes on ice and irradiated with a Panasonic 20W black light-blue lamp (5 cm distance) for 30 min. After photolysis the samples were dialysed against deionized water

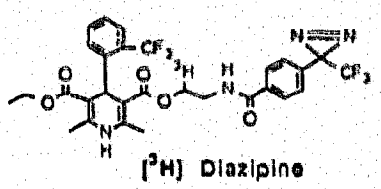

Fig. 1. Chemical structure of [³H]diazipine.

and concentrated with Centricon 30 (Amicon). SDS-PAGE was carried out according to Laemmli [14] using 8% polyacrylamide for the separation gel. The concentrated samples were incubated in the sample buffer for SDS-PAGE containing either 50 mM *N*-ethylmaleimide (NEM) or 50 mM dithiothreitol (DTT) for 2 h at 40°C and loaded onto the gel. After electrophoresis, individual gel lanes were manually cut into 3 mm slices which were solubilized with 30% H_2O_2 (0.75 ml) at 70°C and counted.

3. RESULTS AND DISCUSSION

Binding properties of diazipine and [³H]diazipine were studied in two systems. First, diazipine competitively blocked the binding of [³H](+)PN200-110 to the WGA-Sepharose purified DHP receptor from rabbit skeletal muscle. The obtained IC_{50} value was 16.3 nM (Fig. 2). Hill coefficient (n_H) was 1.00, suggesting a single binding mode. The value of IC_{50} is comparable to that of (±)PN200-110, 20.4 nM which is obtained by the parallel experiment (Fig. 2).

In the second binding system, [³H]diazipine-bound fraction in the purified receptor preparation was increased in a saturable fashion by increasing the [³H]diazipine concentrations, although the nonspecific binding fraction was fairly high (Fig. 3). Scatchard analysis of the specific binding fraction showed a single binding mode (insert in Fig. 3) and gave the values of K_d = 9.3 nM and B_{max} = 770 pmol/mg of protein. The K_d value obtained was comparable with those of the

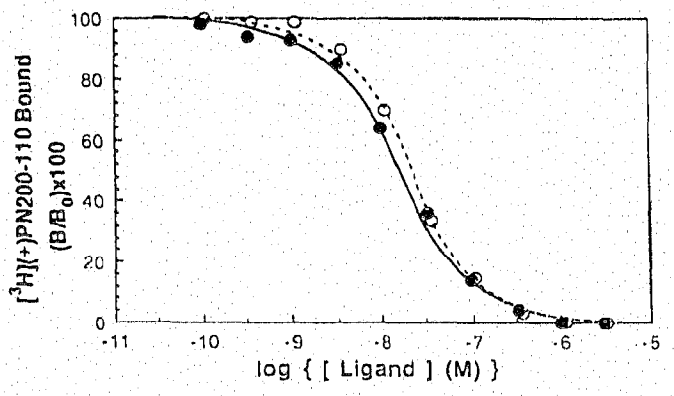

Fig. 2. Inhibition of [³H](+)PN200-110 binding by two photoaffinity ligands of DHP. WGA-purified calcium channel preparations from rabbit skeletal muscle (1.9 μg of protein) were incubated with 3.4 nM [³H](+)PN200-110 in the presence of the indicated concentrations of unlabeled diazipine (●), or PN200-110 (○).

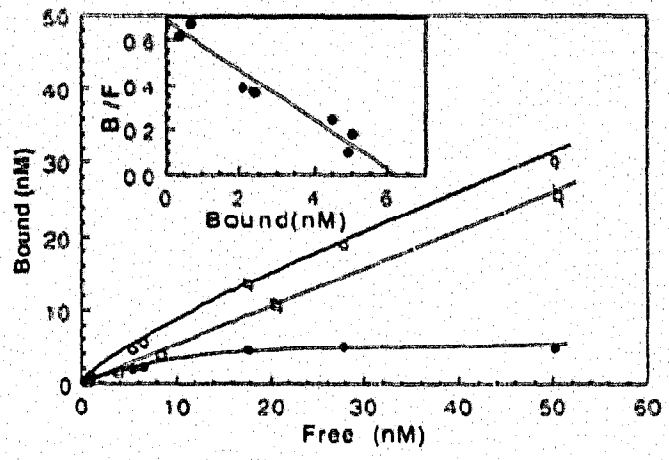

Fig. 3. Saturation isotherm of [³H]diazipine binding. [³H]Diazipine (total concentration 1–80 nM) was incubated with 4 μg of the WGA-purified protein. Total binding (○), nonspecific binding (□), and specific binding (●) are shown. Data points are means of duplicate determinations. The specific binding components were analyzed by a Scatchard plot (depicted in insert) and resulting in a K_d of 9.3 nM and B_{max} of 6.16 nM (or 770 pmol/mg protein).

conventional dihydropyridines, e.g. racemic [³H]azidopine and a biologically active enantiomer [³H](+)PN200-110, 10.5 nM and 5.2 nM respectively, which were separately determined to the same preparation. A fairly high level of nonspecific binding of [³H]diazipine to the purified receptors might be explained in terms of the more hydrophobic character of the compound since it was retained much longer (15 min) on a C18 reversed phase column of HPLC than either azidopine (12 min) or PN200-110 (9 min).

However, this nature is not an obstacle when [³H]diazipine is applied to the photoaffinity labeling of the receptor. As shown in Fig. 4, the reagent was specifically incorporated into the 150 kDa polypeptide, and any of other polypeptides in the preparation which were apparently seen in the silver-stained gel of SDS-PAGE, were not labeled at all. The specific manner of the incorporation was also demonstrated by another experiment where the photoincorporation was completely blocked in the presence of excess amount of PN200-110, one of the typical and specific reversible dihydropyridine ligands for the receptor.

The 150 kDa polypeptide labeled with [³H]diazipine did not change its size on SDS-PAGE after reducing with dithiothreitol (Figs. 4,5), as in the case of [³H]azidopine (Fig. 5). It has been established that the 150 kDa polypeptide labeled with [³H]azidopine is the α-1 subunit of the L-type calcium channel [2,3]. Therefore, [³H]diazipine also labels the α-1 subunit (dihydropyridine receptor) and we conclude that diazipine is a new photoaffinity probe for the dihydropyridine binding site of the calcium channels.

Photoincorporation efficiency of [³H]diazipine (Fig. 5a) is 2 times as high as that of [³H]azidopine

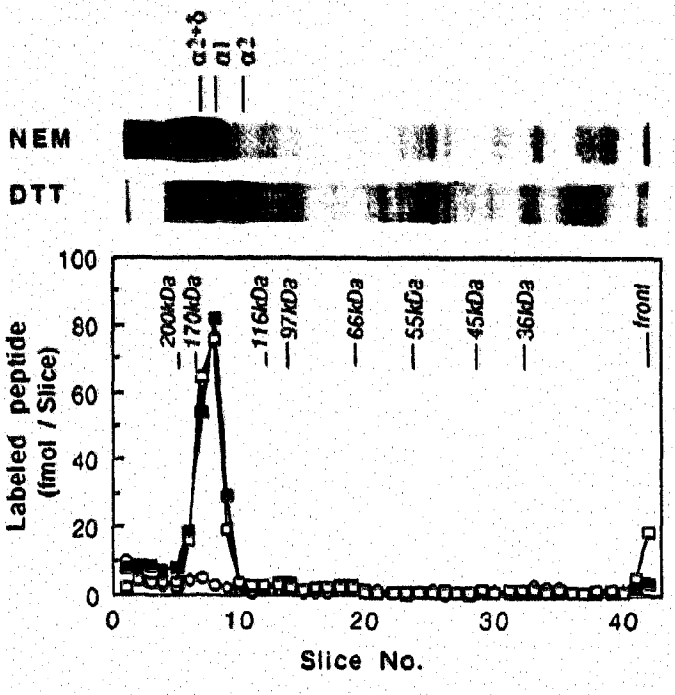

Fig. 4. Photoaffinity labeling of the DHP receptors with [^{3}H]diazipine. The WGA-purified DHP receptors were incubated with 11 nM of [^{3}H]diazipine (square symbol), or 11 nM [^{3}H]diazipine plus 10 μM (±)PN200-110 (circle symbol) for 1 h at 25°C, and the mixture was then photolyzed. After dialysis and concentration, the photolyzed sample (10.5 μg protein or 8 pmol of DHP binding site) was treated either with 50 mM NEM (open symbol) or with 50 mM DTT (filled symbol) and electrophoresed. Silver-stained protein profiles are shown in the upper panel. In the gel slices 6–9, radioisotope was specifically incorporated either in NEM or DTT treatment. M_r standards are shown (myosin, 200 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; bovine serum albumin, 66 kDa; glutamate dehydrogenase, 55 kDa; ovalbumin, 45 kDa; lactate dehydrogenase, 36 kDa).

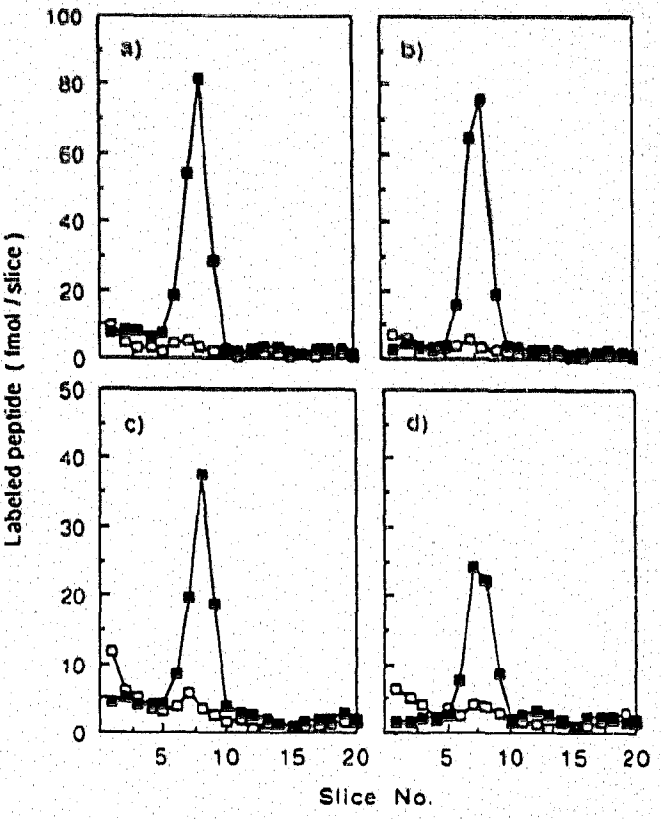

Fig. 5. Comparison of the photolabeled DHP receptor proteins with [^{3}H]diazipine and [^{3}H]azidopine. The receptor proteins, 30% of which were bound reversibly with [^{3}H]diazipine (a, b) or [^{3}H]azidopine (c, d) were photolyzed. The photolyzed samples (10.5 μg protein) were treated either with NEM (a, c) or with DTT (b, d) and electrophoresed as described. The open symbols show the samples photolyzed in the presence of 10 μM (±)PN200-110. Radioactivity in the gel slices up to 20 are shown here; no radioactivity was observed in the following gel slices.

(Fig. 5c), although both of the compounds are estimated to bind reversibly to a similar extent (30%) of the DHP receptors before irradiation. Under the conditions of irradiation, 8% of the bound [^{3}H]diazipine was photoincorporated. Moreover, the photoincorporated amount of [^{3}H]diazipine into the α-1 subunit of the calcium channel did not change after the DTT reduction, as shown in Fig. 5 (a,b). This is in clear contrast to the result of [^{3}H]azidopine that the photoincorporated amount decreased by 33% (Fig. 5c,d), or even more as demonstrated previously [15,16]. These results indicate that the reactive species, phenylcarbene, which is generated from the phenyldiazirine group of diazipine, forms more stable photoproducts against DTT reduction than the phenylnitrene from the phenylazide group of azidopine. It is worth noting that the chemical instability of nitrene-derived photoproducts under acidic conditions may also hamper the identification of the labeled sites sometimes [8,17,18].

In conclusion, diazipine has considerable advantages over conventional azidopine as a photoaffinity probe for L-type calcium channels: higher yield of photoincorporation, forming more stable photoproduct(s), although binding affinity is similar. It will now provide us a more suitable tool to identify the binding site of the dihydropyridine in the calcium channels.

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