CHEMICAL IDENTIFICATION OF BINDING SITES FOR CALCIUM CHANNEL ANTAGONISTS[+]

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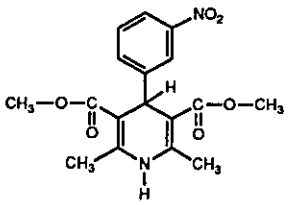
Abstract - Binding sites of three typical calcium channel antagonists including heterocyclic 1,4-dihydropyridine and benzothiazepines, and phenylalkylamines, have been identified within the primary structures of calcium channels by photoaffinity labeling technique. We briefly review the results and discuss the future prospect.

1. Introduction

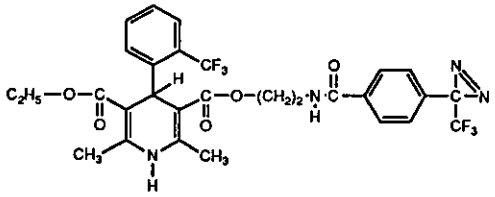
L-type calcium channels are complex oligomeric membrane proteins that regulate voltage-dependent influx of calcium into excitable cells.[1] They represent one of the key components of excitation-contraction coupling machinery in the muscle cells.[2] They are also the receptors for drugs called calcium antagonists or calcium channel blockers.[3] These drugs play an important role in the treatment of many different cardiovascular disorders, particularly hypertension and angina pectoris.[4] Among a number of compounds which act as the calcium channel blockers, 1,4-dihydropyridines (DHPs), phenylalkylamines (PAAs), and benzothiazepines (BTZs) are known as typical three classes of the calcium antagonists. All of the three classes of calcium antagonists, in spite of their different chemical structures including heterocycles (Figure 1), specifically bind to the α1 subunit of the L-type calcium channels and prevent calcium influx into the cells. Binding studies using radioisotope-

[+]Dedicated to the memory of late Professor Yoshio Ban

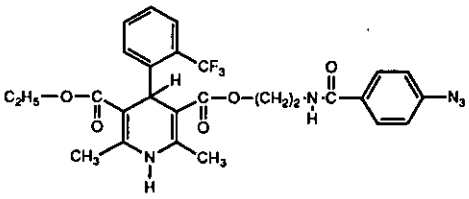
1,4-Dihydropyridines



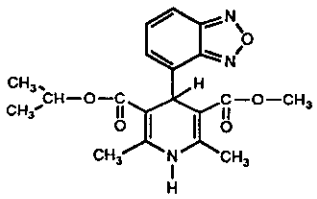
Nitredipine



Diazipine

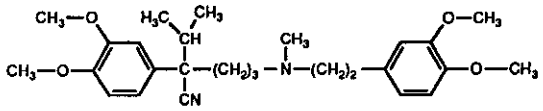


Azidopine

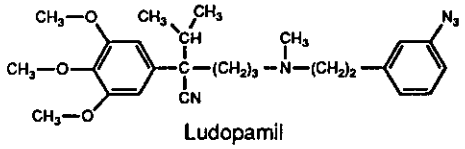


Isradipine

Phenylalkylamines

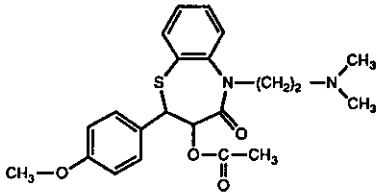


Verapamil

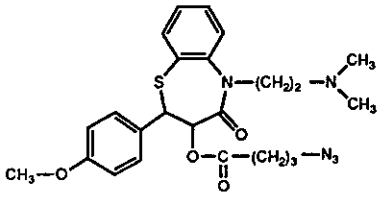


Ludopamil

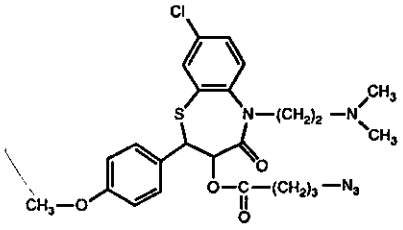
Benzothiazepines



Diltiazem



Azidobutyryl diltiazem



Azidobutyryl clentiazem

Figure 1. Representative structures of three types of calcium channel antagonists which include those for photoaffinity labeling.

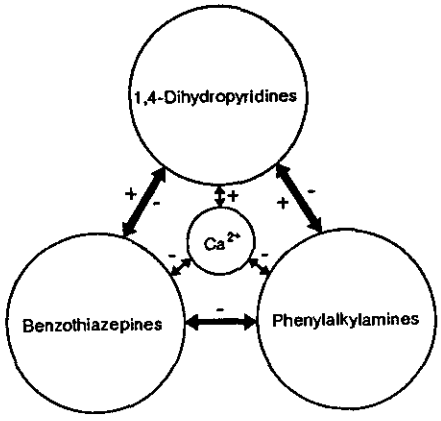


Figure 2. *Allosteric interactions of three typical calcium channel antagonists.* (Redrawn from ref. 5 with modification).

labeled drugs suggest that the binding sites of the three typical drugs are different but they allosterically interact each other,[5] as shown in Figure 2 .

Progresses of ion channel studies in the past decade, owing largely to the molecular biological techniques, have made possible to elucidate the primary structures of ion channels: sodium channel from electric eels as the first example,[6] followed by sodium channels from other tissues, potassium channels and calcium channels as well.[1] Therefore, it is intriguing to identify the primary structures of the binding sites for the calcium antagonists, with the respect of the structures relevant to the integral channel function of calcium ion permeation. Chemical identification of the binding sites for the drugs of typical three classes have been achieved recently by applying photoaffinity labeling techniques. We briefly review the results and describe future prospects based on the current understanding of interactions between the calcium channels and their drugs.

2. Binding sites for the dihydropyridines

DHPs are one of the chemical groups of calcium channel blockers and are among the most useful ligands to identify the L-type calcium channels even in broken cell preparations. Recent electrophysiological studies have indicated that DHP acts on calcium channels only when they are applied to the extracellular compartment.[7,8] These data suggest that the DHP binding site is either located at the extracellular portion of the $\alpha 1$ subunit or is accessible only from the outside of the cell. Chemical identification of the DHP binding site was first accomplished[9] by photoaffinity labeling of

the purified skeletal muscle calcium channels using diazipine (Figure 1), a new DHP analog with phenyldiazirine moiety as a photosensitive group.[10-12]

By irradiation, phenyldiazirine generates phenylcarbene as reactive intermediate, life time of which is considered to be much shorter than the corresponding phenylnitrene of another reactive intermediate.[13] In another word, phenylcarbene is generally expected to be more reactive than the phenylnitrene. This is true in photolabeling of calcium channels using diazipine, a DHP analog of phenylcarbene precursor, when we compared with azidopine, another DHP analog of phenylnitrene precursor.[10,12] Under identical conditions, diazipine can covalently label the calcium channel as 2.5 times much as azidopine, although binding affinity is almost identical in both compounds. In addition, the photolabeled sample by diazirine was stable to dithiothreitol treatment, while one third of the incorporated radioactivity was liberated from the sample photolabeled by azidopine.[10,12]

Photolabeled sites of the skeletal muscle calcium channels have been determined by mapping of labeled peptide fragments of $\alpha 1$ subunit with anti-peptide antibodies that are directed to the particular sequence of the calcium channel (Figure 3). Trypsin cleavage of the photolabeled $\alpha 1$ subunit results in two separate labeled peptide fragments from transmembrane regions of repeats III and IV. One of the fragment of 3 kDa can be recognized by antibody against the sequence 1011-1026, while the other fragment of 6.2 kDa is recognized by antibody against 1338-1351.[9] The photolabeling results show that the DHP binding site is formed by two sites: the extracellular location of the connecting loop between transmembrane segments of S5 and S6 in repeat III, and the extracellular end of the transmembrane segment S6 in repeat IV, as shown in Figure 4.

It was succeedingly reported[14] that two extracellular regions adjacent to the IIIS6 and IVS6 were also identified as the photolabeled sites with another DHP, isradipine (PN200-110). The labeled fragment for the IIIS6 region in this case was 7.3 kDa (sequence 1023-1088) which was just *C*-terminal adjacent to the 3 kDa fragment (sequence 989-1022) photolabeled with diazipine as described above. Since isradipine, unlike diazipine or azidopine, has no spacer arm which connects the 1,4-dihydropyridine ring with the photosensitive group of phenyldiazirine or phenyl azide at ~ 14A length in diazipine or azidopine, respectively, it can be implied that isradipine labeled rather a core region of the binding site.[13]

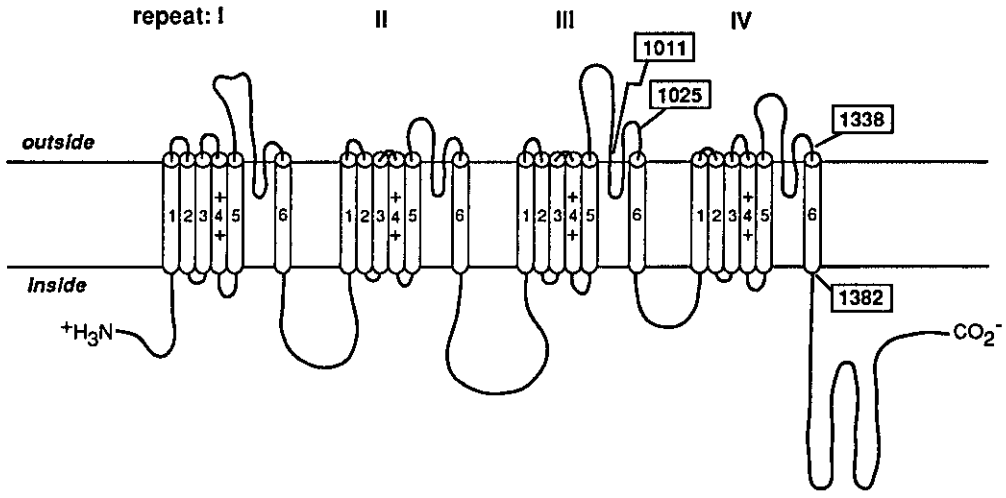


Figure 3. A proposed membrane topology of the α1 subunit of L-type calcium channel. Recognition sites of four anti-peptide antibodies against amino acid sequences begining at numbered residues are also shown.

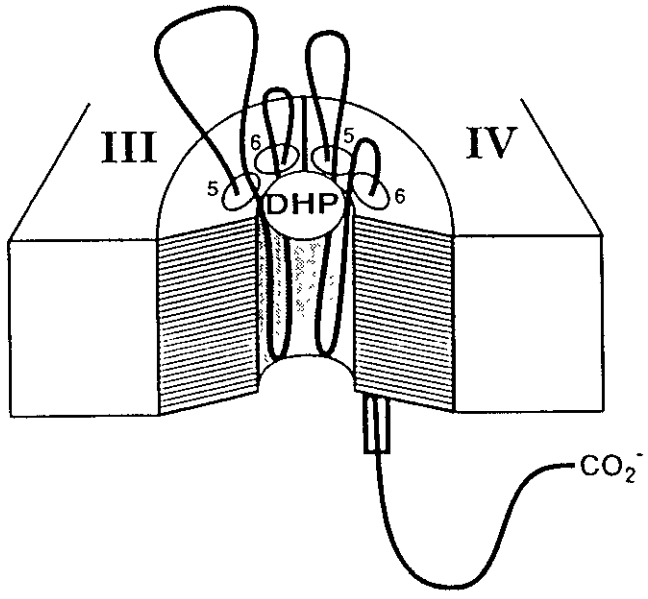


Figure 4. A model for DHP binding on the L-type calcium channel. (Redrawn from ref. 9).

A previous paper by Regulla *et al.*[15] reported that the photolabeled fragments by azidopine were located hydrophilic intracellular parts near *C*- and *N*-terminal regions. They analyzed the reversed-phase hplc-purified peptides by microsequencing procedures. However, if DHP binding site is strongly hydrophobic as expected, it is ikely that its component peptides cannot be easily recovered from hydrophobic reversed-phase hplc column. By contrast, the results obtained from the antibody mapping are consistent with previous pharmacological and physiological observations that DHP acts only when they are applied to the extracellular compartment.[8,9] Therefore, we can conclude that two extracelular located regions near transmembrane contribute the DHP binding site.

3. Binding sites for the phenylalkylamines

Identification of the PAA binding site on skeletal muscle calcium channels was achieved[16] by photoaffinity labeling with ludopamil (LU49888), a photoreactive phenylazide derivative of PAA (Figure 1). The reagent was specifically incorporated into the α1 subunit of the calcium channel and localization of the labeled site was analyzed by the peptide mapping with site-directed antibodies. The results showed that the peptide of 42 amino acid residues extending 1349-1391 which includes transmembrane segment IVS6 and several adjacent intracellular and extracellular amino acid residues was identified as the labeled fragment. Taken together with the observation that quaternary phenylalklyamines reach their binding sites only from the intracellular surface of the membrane,[17] it is implied that the binding site for PAAs including ludopamil consists of the intracellular end of IVS6.

4. Binding sites for the benzothiazepines

Location of the BTZ binding site remained to be solved as the last target to identify the three typical calcium channel antagonists and recently it has been revealed partly by photoaffinity labeling.[18] Initial studies of photolabeling were done by using [^{3}H]azidobutyryl diltiazem (Fig. 1), a photosensitive BTZ. It labeled the α1 subunit of calcium channel as DHP and verapamil analogs but the patterns of labeled peptide fragments were different each other, suggesting the photolabeled sites were distinct in three calcium antagonists.[19] The photolabeled sites has been identified since a new reagent [^{3}H]azidobutyryl clentiazem (Figure 1) was employed.[18]

The new ligand reagent exhibits higher affinity for calcium channel preparations than those of [^{3}H]azidobutyryl diltiazem and diltiazem itself. It enables to photolabel the channel protein (α1 subunit) more selectively. Localization of the photolabeled sites was carried out similarly as the DHP work, by mapping of labeled peptide fragments with site-directed anti-peptide antibodies. Lysyl endoprotease digestion generated several labeled fragments containing 12 kDa as one of the major components. The 12 kDa fragment was recognized by two antibodies against sequences 1338-1351 and 1381-1399, indicating that the labeled site is located in region(s) near transmembrane segment IVS6. Additional results from trypsin cleavage which gave smaller fragments showed that the labeled site was distinct from those by diazipine (DHP) and ludopamil (PAA), even though they label regions near IVS6 as described above. Probable labeling site is located in the extracellular side, which is consistent with the recent electrophysiological results using quaternary benzothiazepines[20] and benzoazepines.[21] Although further studies are required to identify the binding sites for BTZs more in detail, this is the first demonstration that it is composed of extracellular region of IVS6, but at a site different from DHPs and PAAs.

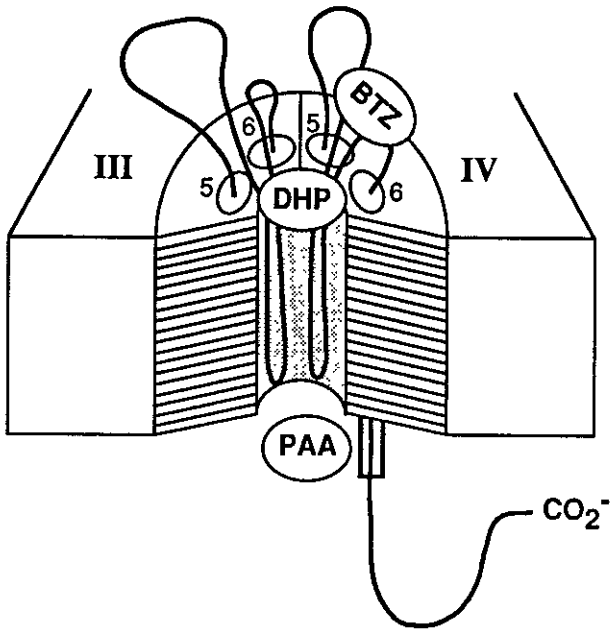


Figure 5. A proposed binding model for the three typical antagonists on the repeat III and IV of the L-type calcium channel.

4. Conclusion and future prospect

In the recourse of the photoaffinity labeling technique, we now get an overview of the binding sites for three typical calcium antagonists within the primary structure of skeletal muscle calcium channel. Their locations are illustrated as Figure 5. DHP binds the extracellular site composed of the IIIS5-IIIS6 loop and the extracellular mouth of IVS6, and BTZ binds another extracellular site, part of which is composed of IVS5-IVS6 loop. By contrast, PA binds intracellular site which consists of the intracellular mouth of IVS6. These findings, not only explain the pharmacological allosteric bindings among the three calcium antagonists (Figure 2) more in detail on the basis of the calcium channel structure, but also shed light on the molecular entity of extracellular and intracellular constituents of channel pore to which the calcium antagonists bind and block the calcium ion permeation.

In addition, the chemically identified sites for the three antagonists are highly conserved in amino acid sequences among L-type calcium channels from skeletal muscle, heart and neurons.

This finding may provide ideas to modify or create new calcium antagonists with different pharmacological characteristics. Minor modifications of these basic drug structures, for example, might yield similarly selective, efficient antagonists of other calcium channel subtypes. Computer-assisted drug designs have been advanced recently and payed much of attention. The designs are greatly helped by three- dimensional structural information of drug receptors. In this respect, three-dimensional elucidation of the calcium channel, the channel-drug complex in particular, is awaited.

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