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STRUCTURAL ANALYSIS OF THE CALCIUM CHANNEL BY PHOTOAFFINITY LABELLING AND LIMITED PROTEOLYSIS

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The L-type calcium channel of rabbit skeletal muscle triads, purified from digitonin extracts, was photolabelled with the dihydropyridine (+)[^{3}H]PN 200-110. This photolabelled form was then subjected to limited proteolysis with papain and staphylococcus V-8 protease and analyzed by polyacrylamide gel electrophoresis. In the absence of proteolysis, the photolabelled channel was represented by a single protein with an apparent molecular weight of 160 kDa in the presence or absence of reducing agents. Following proteolysis, numerous photoadducts were observed with smaller molecular weights. The V-8 protease digestion pattern indicated that photoinsertion occurred in at least two distinct domains of 33 and 28 kDa. Papain digests were more extensive, generating smaller fragments of 28 and ca. 10 kDa. The results suggest that at least two distinct regions of the calcium channel interface at or near the dihydropyridine binding site, and that the binding site for these calcium antagonists resides within the channel proper, thereby modulating calcium influx.

The primary structure of the calcium antagonist receptor of rabbit skeletal muscle, identified by photoaffinity labelling (1,2), has been deduced (3). The sequence homology of this protein with the rat sodium channel protein suggests that this receptor is itself an ion channel (3). This is further supported by a recent finding that monoclonal antibody to the dihydropyridine (DHP) receptor inhibits calcium currents (4). One can therefore assume that calcium antagonists (or calcium channel blockers) act directly upon the L-type voltage-dependent calcium channel to block calcium influx. It is of interest, therefore, to define the region(s) of antagonist binding within the channel structure to explain this mechanism of gating.

While 3-D structural information is lacking regarding these ion selective pores, comparisons have been made with related proteins where such aspects have been studied (5,6,7). These relationships suggest that the calcium channel is composed of four homologous domains that constitute the channel infrastructure and that the unit size of these domains is approximately 30 kDa (3). The results presented here indicate

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that photoaffinity labelling of the calcium channel by a dihydropyridine antagonist results in the covalent modification of at least two transmembrane domains of its primary structure. It is suggested that the binding site for these calcium antagonists resides within the channel proper and thereby modulates channel activity.

Materials and Methods

Reagents The radioisotope (+)[^{3}H]PN 200-110 (85Ci/mMol) was from Amersham (Arlington Heights, IL). Wheat germ lectin-Sepharose, papain, and staphlycoccus V-8 protease were from Sigma (St. Louis, MO). All other chemicals were reagent grade except sodium dodecylsulfate (SDS, sequenol grade, Pierce, Rockford, IL), glycine, N,N'-diallyltartardiamide (DATD) and acrylamide (electrophoresis grade, Bio-Rad Laboratories, Richmond, CA). Aqueous counting scintillant (ACS) was from Amersham. (±)[^{1}H]PN 200-110 was kindly provided by Dr. Leonard Gonasun (Sandoz Research Institute, Sandoz, Inc., E. Hanover, N.J.)

Membrane Preparation Membranes were recovered from freshly dissected rabbit (New Zealand White) back and hind leg skeletal muscle according to (8). Purified membranes were analyzed for protein (9), stored in 20mM TRIS-HCl at -70°C and used within two weeks of isolation. Equilibrium binding of [^{3}H]1,4-dihydropyridine antagonists was determined (10), and scintillation counting conducted for 10 min. per sample in a Searle Delta 300 Liquid Scintillation System.

Channel Purification and Photolabelling Calcium channel purification from digitonin solubilized membranes was accomplished by successive column chromatography using WGA-Sepharose, DEAE-Sephadex and second WGA-Sepharose as reported previously (11). Photolabelling was carried out after the first WGA-Sepharose column step. Sample was incubated with 20 nM [^{3}H]PN 200-110 at 23°C for 60 min. and photolyzed (60 sec) at 4°C in an annular quartz chamber (2mm path length) surrounded by eight 300nm lamps (14 watts per lamp; The Southern New England Ultraviolet Co., Hamden, CT) at a distance of ca. 4 cm. Photolabelled samples were purified through the DEAE-Sephadex column and second WGA-Sepharose column and then subjected to peptide mapping.

Electrophoretic Analyses and Peptide Mapping Purified channel fractions were resolved in acrylamide slab gels (1.5mm) as previously described (12). Gels were stained with Coomassie Brilliant Blue R-250 and/or sliced for scintillation counting. Lanes from the top of the resolving gel through to the dye front were excised and sliced (3mm) and the slices dissolved in 1.2 ml 2% periodic acid for 15 min. at 23°C. Direct gel slicing (no staining) required neutralization of each gel slice with 1 ml 5% glacial acetic acid prior to dissolving with 200 μl 12% periodic acid. Vials were counted in 20 ml ACS for 10 min. Peptide mapping of photolabelled channel fractions were conducted as described (13). Solution digests of the photolabelled channel were conducted in 0.5% SDS at 23°C and terminated with the addition of 4x SDS sample buffer containing n-ethyl-maleimide (1 mg/ml final concentration) or β-mercaptoethanol (8% final concentratation). Molecular weight assignments were based on standards obtained from Sigma (kits MW-SDS-70L/200).

Results and Discussion

It has been established by direct labelling as well as drug competition studies (14) that for each of the three classes of calcium channel antagonist drugs, namely, the 1,4-dihydropyridine (DHP), phenylalkylamines, and benzothiazepines, there is a separate binding site on the receptor molecule. The binding to one site is regulated allosterically by the occupation of the other sites (14). In the present study, crude triad membranes isolated from rabbit skeletal muscles were analyzed by equilibrium binding with [^{3}H]PN 200-110 (^{3}[H]PN). The Scatchard transformation of these binding curves for a typical preparation yielded a B_{max} of 20 pmol/mg protein and a K_d = 0.2nM. The results are consistent with the

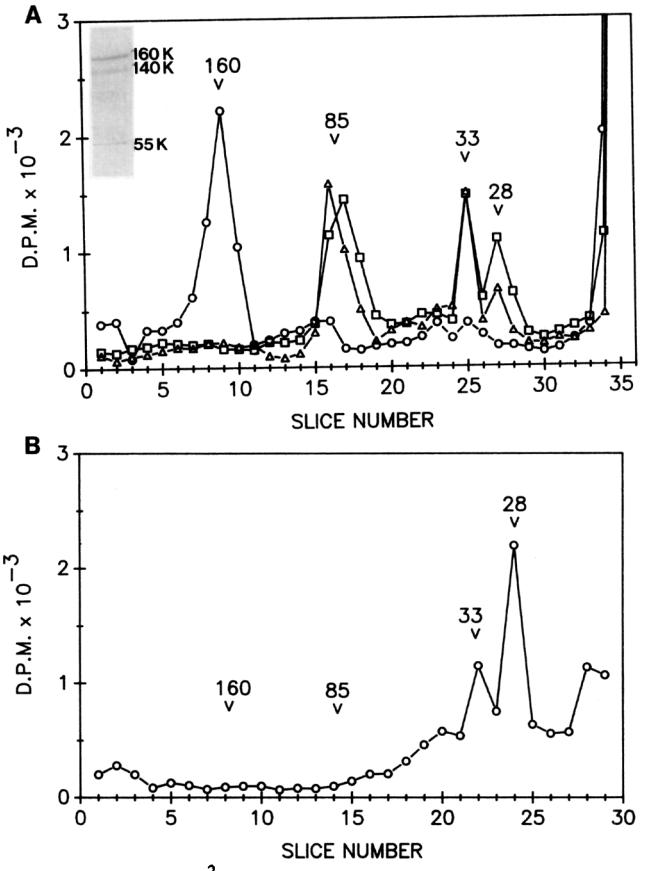

Fig. 1 (a) Solution digests of the ^{3}[H]PN photolabelled calcium channel using V-8 protease. Samples containing 6 μg protein were incubated with or without protease for 60 min., stopped by the addition of 0.1mM PMSF in the 4x SDS sample buffer containing N-ethyl maliemide (see materials and methods) followed by electrophoresis in a 7-15% SDS-DATD gel. The gel was sliced and counted without staining: (○) no protease, (△) 5 μg V-8 protease, and (□) 10 μg V-8 protease. Inset: coomassie-stained gel of 3 μg of protein recovered from the second WGA-Sepharose fractionation (electrophoresis in the presence of β-mercaptoethanol). (b) An equivalent sample digested with 10 μg V-8 protease and treated with 8% β-mercaptoethanol. Numbers above arrowheads indicate the apparent molecular weights of photoadducts (in kilodaltons).

suggestion that the stereospecific binding of DHP class of drugs to calcium channel is on a single domain of the receptor molecule (14, 15). Photolabelling of these preparations with 3[H]PN (not shown) indicated that the major specifically labelled protein has an apparent molecular weight of 160 kDa and is in agreement with values reported by others (1,2).

The protein recovered from the digitonin-solubilized triads following WGA-Sepharose chromatography and DEAE-Sephadex fractionation is represented in Figure 1a (Inset). In the presence of β-mercaptoethanol, the major coomassie-staining proteins exhibit the apparent molecular weights of 160 kDa, 140 kDa and 55 kDa corresponding to the α_1, α_2, β subunits reported by others (15). Thus the result has confirmed that the purified calcium antagonists receptor is an oligomeric protein (16). At the present, it is not clear whether α_2, β and γ proteins are contaminants or true subunits of the calcium channel

complex (15, 16). When samples of the first WGA-Sepharose-specific fraction (see materials and methods) were photolabelled with ^{3}[H]PN and further purified by DEAE-Sephadex and the second WGA-Sepharose column, virtually all of the protein photoinsertion products were associated with the 160 kDa, α_1 component, as shown in Figure 1a (-○-). Unlike the α_2 band, the migration of this α_1 species on SDS gel was unaffected by treatment with β-mercaptoethanol (not shown). These are typical characsteristics of the purified DHP receptor and are in agreement with reports indicating that only one single polypeptide (the α_1 subunit) carries the drug receptor domains, the regulatory phosphorylation sites and the calcium conducting unit (15, 16).

In order to investigate the region(s) of this photoinsertion within the calcium channel, photolabelled samples prepared as above were subjected to limited proteolysis in 0.5% SDS (13) by papain or staphylococcus V-8 protease and then analyzed by SDS polyacrylamide slab gel electrophoresis. V-8 protease digests of the photolabelled channel were resolved on a 7-15% DATD gel, under the non-reducing condition, sliced and counted. The results indicate that during the initial stages of these digestions with low concentration of protease (5 μg), the 160 kDa protein was completely degraded into three photolabelled fragments of Ca. 85 kDa, 33 kDa and 28 kDa in size (Fig. 1a -△-). Digests at higher protease concentration (10 μg) resulted in a small decrease in the 85 kDa form and a corresponding increase of the 28 kDa form while the 33 kDa fragment appeared resistant to further degradation (Fig. 1a -□-). When the same digests containing this 85 kDa product were electrophoresed under reducing condition (in the presence of β-mercaptoethanol, Fig. 1b), this form was converted to the 28 kDa form with the 33 kDa form remaining intact. Thus the 33 kDa species is different from the 85-, and 28- kDa species, regarding the sensitivities toward V-8 protease as well as β-mercaptoethanol. These results suggest that the 33 kDa and 85 kDa forms represent two independently photolabelled products and that the 85 kDa fragment may be composed of two to three fragments of 28 kDa coupled by one or more disulfide bonds.

An alternative procedure was used to analyze digestion with V-8 protease. Samples of the photolabelled calcium channel were further purified and concentrated by SDS-PAGE in a 5-15% DATD gel in the presence of N-ethyl-maleimide. These gels were then stained, the appropriate 160 kDa bands cut out and subjected to re-electrophoresis on a 7-15% DATD gel with increasing concentrations of V-8 protease in the overlay solutions (Figure 2). In these experiments, intermediate forms of 68 kDa and 45 kDa were observed in addition to the 33 kDa and 28 kDa forms seen in comparable solution digests (see Fig. 1a). Again the 33 kDa form here was resistant to V-8 protease digestion (note the complete overlap of the 33 kDa peak at 0.5 μg and 1.0 μg protease concentration), while the generation of 28 kDa form continued at higher protease concentration. Thus the 33 kDa and the 28 kDa are two independently labelled species.

When the photolabelled preparation was subjected to papain digestion for various times, samples were resolved on a 15% DATD gel and analyzed as above. In these experiments (Figure 3), photolabelled fragments of ca. 80 kDa, 55 kDa, 45 kDa, 28 kDa and 10 kDa were

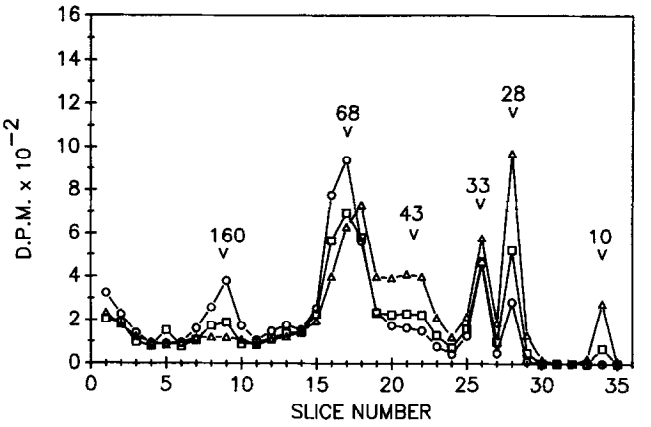

Fig. 2 Digestion of ^{3}[H]PN photolabelled calcium channel bands after re-electrophoresis. Coommassie stained bands recovered from 5-15% SDS-DATD gels where 10 μg aliquots of protein were applied to a 7-15% SDS-DATD gel and followed by digestion with increasing amounts of V-8 protease within the stacking gel: (○) 0.5 μg protease, (□) 1.0 μg protease and (△) 2 μg protease. The gel was sliced and counted without staining. Numbers above arrowheads indicate the apparent molecular weights of photoadducts (in kilodaltons).

apparent during the course of digestion. As was apparent with forms observed with V-8 protease digestion, two independent regions of photoadduction were apparent and were represented by the 28 kDa and 10 kDa forms seen in terminal stages of digestion. In these digests, the 28 kDa form appeared related to the 33 kDa form seen in the V-8 protease profiles since it also was clearly refractory to long term digestion. Moreover, the 45 kDa form, degrading to ca. 10 kDa after prolonged papain digestion, had a related product in the V-8 digests (see figure 2).

The summation of these results indicates that during the initial stages of digestion, two independent adducts are formed and are best characterized in the V-8 protease profiles.

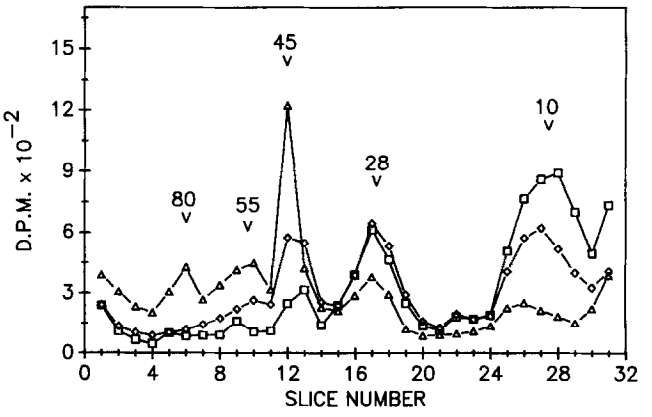

Fig. 3 Solution digests of the ^{3}[H]PN photolabelled calcium channel using papain. Conditions were similar to those of fig. 1 except that samples were resolved on a 15% SDS-DATD gel. Photolabelled samples were mixed with 2 μg of papain and digests carried out for (△) 5 min., (◇) 20 min. and (□) 40 min. prior to the addition of the 4x SDS buffer containing N-ethyl maliemide. The gel was sliced and counted without staining. Numbers above the arrowheads indicate the apparent molecular weight of photoadducts (in kilodaltons).

One form, having an apparent molecular weight of 33 kDa, was relatively stable to further digestion. The other form, having an apparent molecular weight of 85 kDa (initially), undergoes degradation to 28 kDa after passing through intermediates of 65 and 45 kDa. The 28 kDa form is not, however, stable to long term digestion and finally degrades to ca. 10 kDa. This 28 kDa species was not observed following digestion with papain, suggesting a higher susceptability with this protease. We believe that these results indicate aspects of higher calcium channel structure and help to illuminate the nature of the DHP binding site.

Comparative analyses of a number of functionally related (channel) proteins indicate a common structural theme (17). Electron microscopic examination or x-ray crystallography of the photosynthetic reaction center (5), the rat liver gap junction protein (6), and the acetylcholine receptor (7) suggest that these proteins are composed of a radial arrangement of cylindrically-shaped membrane-spanning subunits (or domains) with considerable α-helical content. The pore, or channel, is defined by the axial region around which these components are arranged.

Thus, higher structural information for the calcium channel can be inferred from these analogies. These relationships, which have also been employed to describe structural aspects of the sodium channel, suggest that both these ion-selective pores are composed of four internally repeated homologous domains of approximately 30 kDa joined by a string of hydrophilic amino acid residues (3,18). The results presented here are consistent with this proposed membrane topology for the calcium channel and imply that 3-D structural features of the calcium channel persist in the digitonin solubilized state. Fragments of approximately 30 kDa (the labile as well as the stables types) were generated by either V-8 digestion or papain digestion. A comparison to the deduced primary structure (3) also indicates that the sites of V-8 protease susceptibility (at glutamate residues) are primarily (but not exclusively) limited to regions outside these transmembrane domains, while the sites of papain susceptibility (at hydrophobic residues) may include some transmembrane regions. This can explain the result that smaller fragments (28 kDa and 10 kDa) were generated by papain as compared to the corresponding 33 kDa and 28 kDa fragments from the V-8 digests. Thus it is reasonable to assume that these 33 and 28 kDa fragments represent all or part of at least two of these four unit structures. The results suggest that the stereospecific binding of one site by (+)[^{3}H]PN200-110, results in the photolabelling of at least two distinct domains due to the 3-D structure of the Ca^{2+} channel. Indeed, the data from Fig. 1b suggest further that all four domains may be photolabelled, and one of these is more resistant to proteolysis than the other three that are held together by disulfide bonds (the ratio of radioactivity in the 33 kDa peak vs. 28 kDa peak is nearly one to three). It therefore seems possible that the DHP binding site exists within the channel proper or offset from its axial center. There is no doubt that the precise region of DHP binding can be resolved by sequence analysis of the protease digestion fragments reported here. Nevertheless, it appears likely that these calcium

antagonists, and related agonists, act directly within the calcium channel infrastructure and thereby modulate calcium influx.

Acknowledgements

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