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Direct Photoaffinity Labeling of the High Affinity Nitrendipine-binding Site in Subcellular Membrane Fractions Isolated from Canine Myocardium*

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[^{3}H]Nitrendipine and high intensity ultraviolet irradiation have been used to photoaffinity label the protein component of the high affinity nitrendipine-binding site in subcellular membrane fractions from canine cardiac muscle. Irradiation of isolated cardiac membranes in the presence of [^{3}H]nitrendipine resulted in the covalent labeling of a protein component that migrated on sodium dodecyl sulfate-polyacrylamide gels with an apparent molecular weight of 32,000. Incorporation of [^{3}H]nitrendipine did not occur in the absence of irradiation. The photoaffinity labeling of the 32,000-Da protein by [^{3}H]nitrendipine was inhibited by excess unlabeled nitrendipine, nifedipine, or verapamil. EDTA, ATP, and La^{3+}, which are known to reduce high affinity nitrendipine binding, also inhibited the photoaffinity labeling of this membrane protein by [^{3}H]nitrendipine. The 32,000-Da [^{3}H]nitrendipine-labeled protein was found to be enriched in the ryanodine-sensitive fraction of cardiac sarcoplasmic reticulum and absent from the ryanodine-insensitive fraction of cardiac sarcoplasmic reticulum which is known to lack high affinity nitrendipine binding. Therefore, the 32,000-Da photoaffinity-labeled [^{3}H] nitrendipine-binding protein exhibits properties identical to those expected for the protein component of the high affinity nitrendipine-binding site in isolated cardiac membranes.

Cardiac and smooth muscle contraction are initiated and modulated by Ca^{2+} influx across the cell membrane via the slow inward Ca^{2+} channel (1-3). Various organic compounds are known to alter Ca^{2+} influx into the cell and are referred to as Ca^{2+} antagonists or Ca^{2+} channel blockers (4-6). Dihydropyridines are the most potent class of Ca^{2+} channel blockers (5, 6). The dihydropyridine, [^{3}H]nitrendipine, has been shown to bind with high affinity to crude membranes isolated from cardiac and vascular muscles (7, 8). The exact relationship between high affinity nitrendipine binding to isolated membranes and pharmacological effect of nitrendipine on the slow inward Ca^{2+} channel has yet to be determined. A complete understanding of the mechanism of action of nitrendipine on cardiac and smooth muscle requires the identification and characterization of the membrane component of the slow inward Ca^{2+} channel which binds nitrendipine with high affinity. Recently, Venter *et al.* (9) have approached this problem using an affinity analog of nitrendipine with intestinal smooth muscle membranes. We have approached this problem using direct photoaffinity labeling with [^{3}H]nitrendipine and subcellular membrane fractions from canine myocardium which have been well characterized with respect to high affinity nitrendipine binding by Williams and Jones (10). Our results show that [^{3}H]nitrendipine is an excellent natural photoaffinity ligand. Direct photolabeling of isolated cardiac membranes with [^{3}H]nitrendipine has allowed us to identify and characterize a 32,000-Da [^{3}H]nitrendipine-labeled protein which has all the properties expected for the protein component of the high affinity nitrendipine-binding site in cardiac muscle.

EXPERIMENTAL PROCEDURES

Preparation of Cardiac Sarcoplasmic Reticulum and Sarcolemma Membranes—Subcellular membrane fractions were isolated from canine ventricular muscle using the procedures of Jones (11-13) in the presence of the following protease inhibitors: pepstatin A (10 μg/ml), antipain (10 μg/ml), leupeptin (10 μg/ml), chymostatin (10 μg/ml), benzamidine (100 μg/ml), Kallikrein inactivator (500 units/ml), and PMSF[1] (1.0 mM). Crude cardiac membrane vesicles were isolated according to Jones *et al.* (11). Cardiac sarcoplasmic reticulum membrane vesicles were isolated according to Jones and Cala (12) using Ca^{2+} oxalate loading followed by sucrose density gradient centrifugation. Ca^{2+} oxalate loading was also carried out in the presence of protease inhibitors. Subfractions A-E were collected as described (12). Subfractions B, C, and D corresponded to the ryanodine-sensitive sarcoplasmic reticulum vesicles. Subfraction E corresponded to the ryanodine-insensitive sarcoplasmic reticulum vesicles. Highly purified cardiac sarcolemma vesicles were isolated from canine ventricular muscle according to Jones *et al.* (13). All membrane preparations were stored frozen at −70 °C in 0.25 M sucrose, 10 mM histidine (pH 7.4). Protein was determined by the method of Lowry *et al.* (14) using the modification of Peterson (15).

Photoaffinity Labeling of Isolated Membrane Fractions with [^{3}H] Nitrendipine—Subcellular membrane fractions (1.0 mg of protein/ml, final concentration) were equilibrated at 37 °C for 30 min with a saturating concentration of [^{3}H]nitrendipine (10 nM) in 0.5 ml of 0.15 M NaCl, 10 mM Tris (pH 7.4), and 0.5 mM PMSF. Nonspecific labeling was determined by the inclusion of nitrendipine (10^{-6}-10^{-5} M). Samples were photoactivated by high intensity UV light from a 1000-watt mercury arc lamp (Porta-Cure 1000, American Ultraviolet Co.). Exposure was at 4 °C for 20 s at a distance of 25 cm from the lamp. Samples were solubilized by the addition of an equal volume of SDS-gel sample buffer (6% SDS, 125 mM Tris (pH 6.8), 1 mM β-mercaptoethanol, 20% glycerol, and 0.5 mM PMSF) and immediately heated at 100 °C for 1 min.

SDS-Polyacrylamide Gel Electrophoresis—[^{3}H]Nitrendipine labeled membranes (225 μg of protein) were analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli (16) using 3.0-mm thick gradient gels (8-18% acrylamide). Gels were stained with Coomassie blue and then extensively destained in a solution of 10% acetic acid and 5% methanol. [^{3}H]Nitrendipine incorporation was detected by fluorography and/or liquid scintillation counting of gel slices. Gels used for fluorography were treated with Enlightning before drying and then exposed at −70 °C using Kodak XAR-5 film. Gel slices (2.5 mm) were digested overnight at 45 °C in 0.8 ml of 30% H_2O_2 before liquid scintillation fluid was added. Specific activity of [^{3}H]nitrendi-

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[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

pine was determined under the same conditions using unlabeled gel slices and H_2O_2. Radioactivity was measured using a Beckman LS 8100 Liquid Scintillation Counter which was interfaced with a Vax 11/780 computer. Plots of [^{3}H]nitrendipine incorporation *versus* gel slice number were obtained directly from the Vax 11/780 on a Tektronix 4014-1 graphics terminal. Apparent molecular weights were calculated from a graph of relative mobilities *versus* log molecular weight of standard proteins.

Materials—[^{3}H]Nitrendipine (88 Ci/mmol) and Enlightning were obtained from New England Nuclear. Nitrendipine was generously supplied by Miles Laboratories. Nifedipine was generously supplied by Pfizer. Verapamil was generously supplied by Knoll Pharmaceutical Co. Electrophoretic reagents and molecular weight standards were obtained from Bio-Rad. Protease inhibitors (antipain, pepstatin A, chymostatin, leupeptin, benzamidine, and PMSF) were obtained from Sigma. Kallikrein inactivator was obtained from Calbiochem. All other reagents were of reagent grade quality.

RESULTS AND DISCUSSION

Photoaffinity labeling, which usually requires a ligand with a photoreactive group, can be a very powerful tool for identifying and characterizing specific membrane receptors. Certain ligands can be photoactivated directly by irradiation with high intensity UV light (17–20). The fact that the dihydropyridines, including nitrendipine, are light-sensitive compounds, suggested to us that [^{3}H]nitrendipine might be a natural photoaffinity ligand and react covalently with the membrane component of the high affinity dihydropyridine binding site in the presence of high intensity UV light. We have tested this possibility using subcellular membrane fractions from canine myocardium which have been well characterized with respect to high affinity nitrendipine binding (10).

Fig. 1 shows the [^{3}H]nitrendipine labeling profile obtained for the ryanodine-sensitive fraction of cardiac sarcoplasmic reticulum vesicles following high intensity UV irradiation. [^{3}H]Nitrendipine was incorporated into predominantly one protein of molecular weight 32,000 (Fig. 1, *lane 1*). Addition of unlabeled nitrendipine (2×10^{-5} M) to the incubation buffer before UV irradiation greatly reduced the incorporation of [^{3}H]nitrendipine into the 32,000-Da protein (Fig. 1, *lane 2*). In the absence of UV irradiation, no incorporation of [^{3}H]nitrendipine was observed (not shown). Addition of excess unlabeled nitrendipine following UV irradiation did not alter the amount of [^{3}H]nitrendipine found in the 32,000-Da protein (not shown). [^{3}H]Nitrendipine was also incorporated into a diffuse band with an average molecular weight of 12,000 Da (Fig. 1, *lane 1*). An autoradiograph of a high percentage gel that gives better resolution of the low molecular weight proteins has indicated that this diffuse band of radioactivity actually consists of at least two labeled proteins (not shown). Photolabeling of this diffuse band was also sensitive to the addition of unlabeled nitrendipine (Fig. 1, *lane 2*). Removing PMSF from the incubation buffer decreased the intensity of the 32,000-Da protein and increased the intensity of the 12,000-Da band suggesting that at least one protein in the diffuse 12,000-Da band is a proteolytic fragment of the 32,000-Da protein. Another band of radioactivity was found in front of the bromphenol blue tracking dye and was even found in the presence of excess unlabeled nitrendipine (not shown). We believe this band of radioactivity represents free [^{3}H]nitrendipine or nonspecific incorporation of [^{3}H]nitrendipine into membrane phospholipid.

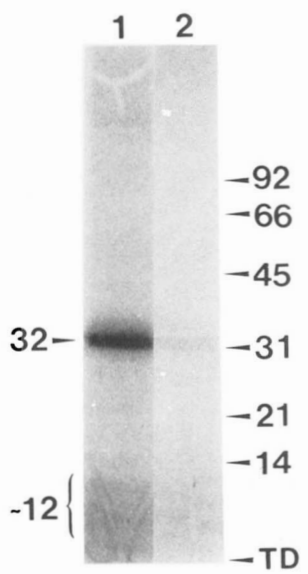

FIG. 1. **Photoaffinity labeling of ryanodine-sensitive sarcoplasmic reticulum vesicles with [^{3}H]nitrendipine (autoradiograph profile).** Ryanodine-sensitive sarcoplasmic reticulum vesicles (1 mg/ml) were equilibrated at 37 °C for 30 min in 150 mM NaCl, 10 mM Tris (pH 7.4), 0.4 mM PMSF with 20 nM [^{3}H]nitrendipine in the absence (*lane 1*) and presence (*lane 2*) of unlabeled nitrendipine (2×10^{-5} M) and then exposed to UV light for 20 s. Samples (225 μg of protein) dissolved in SDS-gel buffer were subjected to SDS-polyacrylamide gel electrophoresis (8–18% gradient gels) as described under "Experimental Procedures." Following staining and destaining, the slab gel was then treated with Enlightning and exposed to x-ray film at −70 °C. *Arrowheads* on the *right* depict the location of molecular weight standards and the position of tracking dye (*TD*). *Arrowheads* on the *left* indicate [^{3}H]nitrendipine-labeled proteins. *Numbers* represent $M_r \times 10^{-3}$.

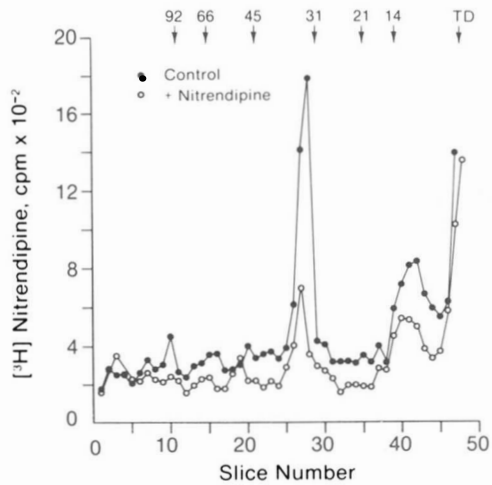

FIG. 2. **Photoaffinity labeling of ryanodine-sensitive sarcoplasmic reticulum vesicles with [^{3}H]nitrendipine (electrophoretic profile).** Ryanodine-sensitive sarcoplasmic reticulum vesicles were photolabeled as described in Fig. 1 in the absence (*solid circles*) or presence (*open circles*) of unlabeled nitrendipine (2×10^{-5} M). Samples (225 μg of protein) were subjected to electrophoresis as described under "Experimental Procedures." Following staining and destaining, gel lanes were sliced in 2.5-mm sections and [^{3}H]nitrendipine incorporation was measured by liquid scintillation counting following solubilization of the gel slices with H_2O_2. *Arrows* depict the location of molecular weight standards and tracking dye (*TD*). *Numbers* represent $M_r \times 10^{-3}$.

The electrophoretic profile of [^{3}H]nitrendipine incorporation obtained for the same labeled membranes used in Fig. 1 is shown in Fig. 2. In absence of unlabeled nitrendipine, one major peak of [^{3}H]nitrendipine incorporation was observed at 32,000 Da along with a broad peak of [^{3}H]nitrendipine incorporation at 12,000 Da. The molecular weight of the diffuse 12,000-Da band was determined using low molecular weight standards (not shown). When aliquots of the same membranes were irradiated in the presence of unlabeled nitrendipine (2×10^{-5} M) the amount of covalently bound [^{3}H]nitrendipine in the 32,000-Da protein was decreased by approximately 70%. The number of high affinity nitrendipine-binding sites photolabeled with [^{3}H]nitrendipine was approximately 110 fmol/mg of ryanodine-sensitive sarcoplasmic reticulum vesi-

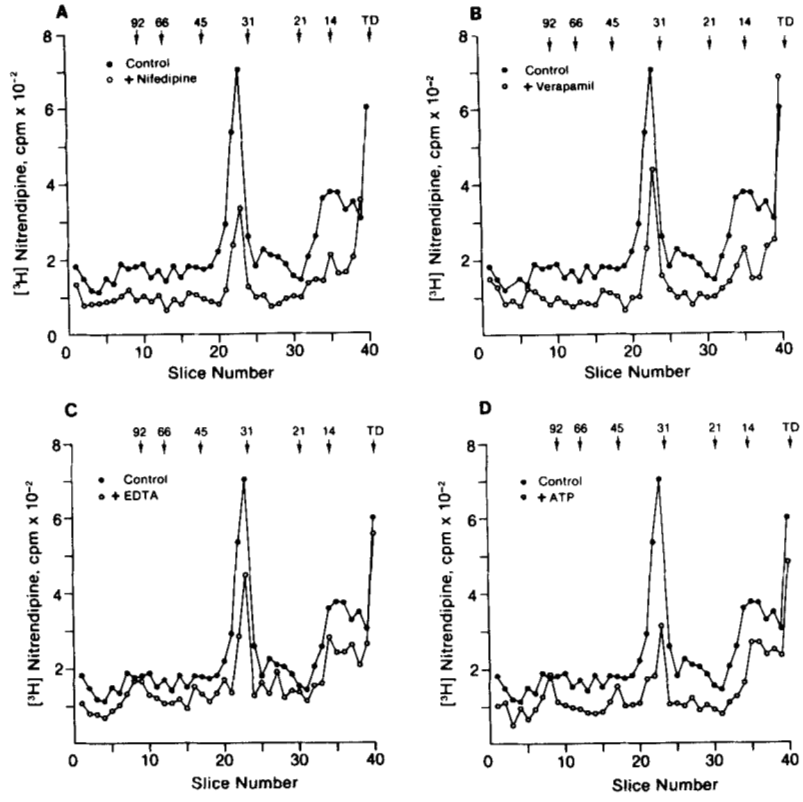

FIG. 3. **Effect of nifedipine, verapamil, EDTA, and ATP on photoaffinity labeling of ryanodine-sensitive sarcoplasmic reticulum vesicles with [^{3}H]nitrendipine.** Ryanodine-sensitive sarcoplasmic reticulum vesicles (0.5 mg/ml) were equilibrated at 37 °C for 30 min in 0.25 M sucrose, 10 mM histidine (pH 7.4), 0.5 mM PMSF with 10 nM [^{3}H]nitrendipine in the absence (*solid circles*) of any additional reagent or in the presence (*open circles*) of *A*, nifedipine (2×10^{-5} M); *B*, verapamil (4×10^{-5} M); *C*, EDTA (10^{-3} M), and *D*, ATP (10^{-2} M). Photolabeling was performed as described in Fig. 1, and samples (112 μg of protein) were analyzed as described in Fig. 2. *Arrows* depict the location of molecular weight standards and tracking dye (*TD*). *Numbers* represent $M_r \times 10^{-3}$.

cles. This was calculated using the ^{3}H counts/min recovered in the 32,000-Da peak in the electrophoretic analysis of the photolabeling. Since the specific activity of [^{3}H]nitrendipine was measured under the same conditions as the ^{3}H counts/min in the gel slices, no corrections were required for quenching. The efficiency of this direct photoaffinity labeling technique was approximately 7%. This was determined by dividing the number of high affinity nitrendipine-binding sites, photolabeled with [^{3}H]nitrendipine, by the reported value of 1.5 pmol/mg for the density of high affinity nitrendipine-binding sites in the ryanodine-sensitive vesicles (10). This is similar to the results of Carter-Su *et al.* (17) for the direct photolabeling of the D-glucose transporter with [^{3}H]cytochalasin B.

The sensitivity of the photoaffinity labeling of the 32,000-Da protein to other Ca^{2+} channel blockers, which are known to reduce the high affinity nitrendipine binding, was tested to show the specificity of the photoaffinity labeling by [^{3}H] nitrendipine. Nifedipine, which is able to compete with nitrendipine for the high affinity dihydropyridine-binding site (6), was able to inhibit the incorporation of [^{3}H]nitrendipine into the 32,000-Da protein (Fig. 3*A*) by a similar extent as unlabeled nitrendipine. Verapamil, which presumably binds to a site which can allosterically regulate high affinity [^{3}H] nitrendipine binding (21), was also able to inhibit the photoaffinity labeling of the 32,000-Da protein by [^{3}H]nitrendipine (Fig. 3*B*).

High affinity nitrendipine binding of isolated membranes can also be inhibited by divalent cation chelators, high concentrations of nucleotides, and heavy metals (22–26). The sensitivity of photoaffinity labeling of the 32,000-Da [^{3}H] nitrendipine-binding protein to the above reagents was also tested to show the specificity of the [^{3}H]nitrendipine incorporation. Fig. 3, *C* and *D* shows the electrophoretic profiles of [^{3}H]nitrendipine incorporation in the absence and presence of 1.0 mM EDTA and 10 mM ATP, respectively. In both cases the incorporation of [^{3}H]nitrendipine into the 32,000-Da protein is inhibited. La^{3+} (0.4 mM) also inhibited the labeling of the 32,000-Da protein by [^{3}H]nitrendipine (not shown). In-

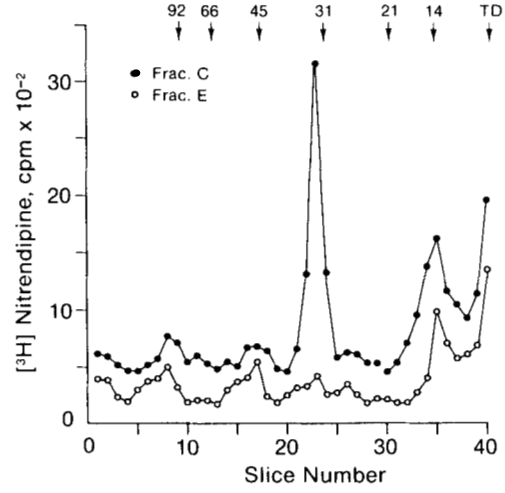

FIG. 4. **Photoaffinity labeling of ryanodine-sensitive and ryanodine-insensitive sarcoplasmic reticulum vesicles with [^{3}H]nitrendipine.** Ryanodine-sensitive (*solid circles*) and ryanodine-insensitive (*open circles*) cardiac sarcoplasmic reticulum vesicles (1 mg/ml) were equilibrated at 37 °C for 30 min in 150 mM NaCl, 10 mM Tris (pH 7.4), 0.5 mM PMSF with 20 nM [^{3}H]nitrendipine. Photolabeling was performed as described in Fig. 1 and samples (225 μg of protein) were analyzed as described in Fig. 2. *Arrows* depict the location of molecular weight standards and tracking dye (*TD*). *Numbers* represent $M_r \times 10^{-3}$.

corporation of [^{3}H]nitrendipine into the 32,000-Da protein was also inhibited if the membranes were first heated at 65 °C for 10 min before labeling (not shown). Thus, we have been able to verify that the covalent photoaffinity labeling of the 32,000-Da protein by [^{3}H]nitrendipine has the same specificity as the high affinity nitrendipine binding to isolated cardiac membranes.

Williams and Jones (10) have found that the ryanodine-sensitive fraction of cardiac sarcoplasmic reticulum is en-

riched in high affinity nitrendipine binding while ryanodine-insensitive sarcoplasmic reticulum vesicles contain little high affinity nitrendipine binding. We have compared the covalent incorporation of [^{3}H]nitrendipine into both types of vesicles in order to further support our identification of the 32,000-Da [^{3}H]nitrendipine-binding protein as the membrane component of the high affinity nitrendipine-binding site. Fig. 4 shows the electrophoretic profile of [^{3}H]nitrendipine incorporation into ryanodine-sensitive and ryanodine-insensitive vesicles. The 32,000-Da photoaffinity labeled [^{3}H]nitrendipine-binding protein was found in the ryanodine-sensitive vesicles and was completely absent from the ryanodine-insensitive vesicles. Specific [^{3}H]nitrendipine incorporation into the diffuse band of approximately 12,000 Da was found in both membrane fractions although it was reduced in the ryanodine-insensitive vesicles. The protein of approximately 12,000 Da labeled in the ryanodine-insensitive vesicles is one of the two labeled in the ryanodine-sensitive vesicles that is unaffected by nitrendipine and therefore is not derived from the 32,000-Da protein. An autoradiograph of a high percentage acrylamide gel has also indicated that this protein is not the protein which is a proteolytic fragment of the 32,000-Da [^{3}H] nitrendipine-labeled protein. The density of specific incorporation of [^{3}H]nitrendipine into the 32,000-Da protein was greatest in the ryanodine-sensitive fraction of cardiac sarcoplasmic reticulum vesicles. Since the ryanodine-sensitive fraction is enriched in cardiac calsequestrin (27), we believe that this fraction is derived from the junctional sarcoplasmic reticulum and probably consists of isolated triads and dyads. Photoaffinity labeling of crude cardiac membranes and highly purified cardiac sarcolemma also resulted in the specific labeling of a 32,000-Da protein by [^{3}H]nitrendipine (not shown). Therefore, it is our present contention that the 32,000-Da [^{3}H]nitrendipine-labeled protein is enriched in the ryanodine-sensitive fraction because this fraction contains transverse tubular membrane vesicles.

Radiation inactivation analysis has indicated that the size of the slow inward Ca^{2+} channel is 278,000 Da in smooth muscle membranes (9) and 210,000 Da in skeletal transverse tubular membranes (28). Thus, it seems unlikely that the 32,000-Da [^{3}H]nitrendipine-labeled protein could be the entire Ca^{2+} channel. The differences in the apparent molecular weight of the high affinity dihydropyridine-binding component observed by us (32,000 Da in canine cardiac membranes) and Venter *et al.* (9) (45,000 Da in guinea pig ileal longitudinal smooth muscle membranes) could be due to the different labeling techniques and/or the different source of membranes. Since the solubilized Ca^{2+} antagonist receptor appears to be a glycoprotein (29), it is also possible that the differences in molecular weight observed are due to different amounts of glycosylation of the membrane protein responsible for the high affinity dihydropyridine binding in cardiac and smooth muscle.

The results of our experiments have shown that [^{3}H]nitrendipine is an excellent photoaffinity ligand for the high affinity nitrendipine-binding site in isolated cardiac membranes. The main advantage of this direct photolabeling with high intensity UV light is that it does not require the synthesis of a ligand with a photoreactive group and therefore it is anticipated that this technique could also be applied to other dihydropyridine compounds. The main disadvantage of this direct photolabeling technique is that efficiency of labeling is low and therefore it requires that the high affinity nitrendipine-binding sites be saturated. But, it should be noted that specific incorporation of [^{3}H]nitrendipine into the 32,000-Da [^{3}H]nitrendipine-binding protein was observed in the presence of 0.5 nM [^{3}H]nitrendipine but the actual counts incorporated were low.

The 32,000-Da [^{3}H]nitrendipine-labeled protein has exhibited all the properties expected for the protein component of the high affinity nitrendipine-binding site and could be the nitrendipine-binding component of the slow inward cardiac Ca^{2+} channel. The exact relationship between the 32,000-Da [^{3}H]nitrendipine-labeled protein and the high affinity binding component of the slow inward Ca^{2+} channel will require further characterization of the 32,000-Da protein.

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