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Structural Characterization of the Nitrendipine Receptor of the Voltage-Dependent Ca^{2+} Channel: Evidence for a 52,000 Dalton Subunit

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Summary: The nitrendipine receptor of the voltage-dependent Ca^{2+} channel purified from rabbit skeletal muscle has been shown to contain four polypeptide components of 175,000, 170,000, 52,000, and 32,000 daltons. Despite the existence of a substantial amount of data on the composition of the nitrendipine receptor, little is known about the relationship between the 175,000 and 170,000 dalton subunits of the receptor and the lower molecular weight components of the receptor. A monoclonal antibody specific to the 52,000 dalton component of the receptor has now been produced. The monoclonal antibody is capable of specifically immunoprecipitating the [^{3}H]dihydropyridine-labeled nitrendipine receptor from detergent-solubilized membranes. Immunoprecipitation experiments with ^{32}P-labeled nitrendipine receptor have demonstrated a tight association between the 170,000 dalton nitrendipine binding subunit and the 52,000 dalton polypeptide of the receptor. Immunoblotting experiments have shown that the 52,000 dalton polypeptide copurifies with the 175,000 and 170,000 dalton subunits of the nitrendipine receptor at all stages of the purification. In addition, the higher molecular weight subunits of the receptor were not labeled by the antibody. Densitometric scanning ofCoomassie blue stained SDS-polyacrylamide gels of the purified nitrendipine receptor has shown that the 175,000, 170,000, 52,000, and 32,000 dalton subunits of the nitrendipine receptor exists in a 1:1:1:1 stoichiometric ratio. In conclusion, we have demonstrated that the 52,000 dalton polypeptide is an integral and distinct subunit of the purified nitrendipine receptor of the voltage-dependent Ca^{2+} channel. **Key Words:** Nitrendipine receptor—Ca^{2+} channel—Rabbit skeletal muscle.

We have recently shown that the purified nitrendipine receptor from rabbit skeletal muscle contains four protein components of 175,000, 170,000, 52,000, and 32,000 daltons when analyzed by SDS–PAGE under nonreducing conditions (1–3). The two high molecular weight subunits of the nitrendipine receptor have been well characterized by biochemical and immunological means. However, little is known about the low molecular mass components of the receptor. The association of these smaller polypeptides with the receptor has been demonstrated only by their presence on SDS–polyacrylamide gels of the purified receptor (1,4–8), their stoichiometry also has not been determined unequivocally, and the low molecular weight peptides have not been reported in certain preparations of purified receptor (9).

METHODS

Monoclonal antibodies against the nitrendipine receptor from rabbit skeletal muscle were prepared as previously described (1). Nitrendipine receptor was purified from skeletal muscle microsomes by solubilization with digitonin and chromatography on wheat germ agglutinin (WGA)-Sepharose followed by chromatography on DEAE-cellulose (1). The specificity of the monoclonal antibodies to the 52,000 dalton subunit of the nitrendipine receptor was determined by immunodot, immunoblot, and immunoprecipitation assays as described (1–3). The methods are summarized by the flow diagrams in each figure.

RESULTS

Structural characterization of the 52,000 dalton subunit of the nitrendipine receptor

A monoclonal antibody (mAb $VD2_1$) specific to the 52,000 dalton component of the nitrendipine receptor of the voltage-dependent Ca^{2+} channel and capable of specifically immunoprecipitating the radiolabeled nitrendipine receptor from detergent-solubilized membranes has been produced (Figs. 1 and 2). Immuno-

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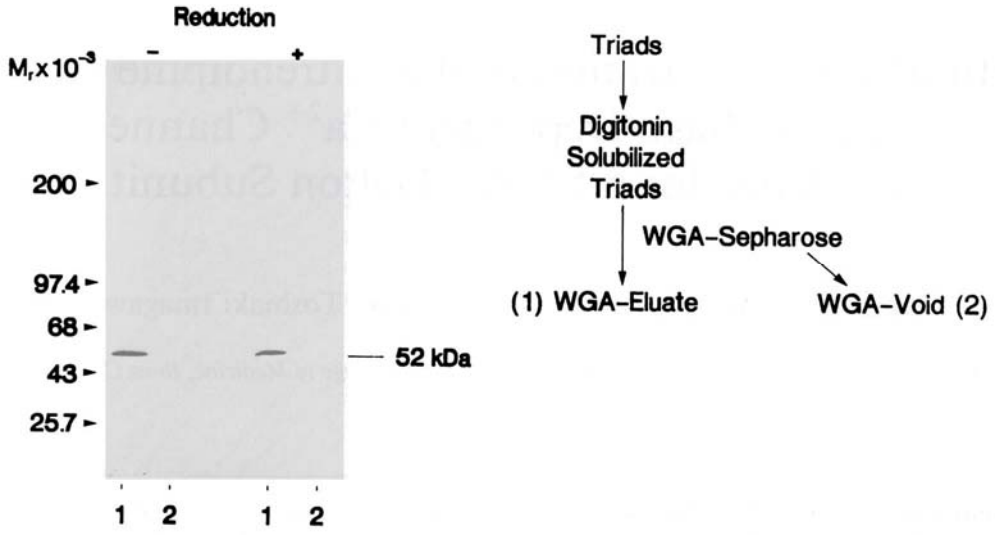

FIG. 1. Immunoblot staining with monoclonal antibody $VD2_1$ (anti-52,000 dalton subunit). Partially purified nitrendipine receptor (lane 1) and the void from WGA-Sepharose (lane 2) were subjected to SDS-PAGE on a 5-16% polyacrylamide gel under reducing (+) and nonreducing (-) conditions and transferred to nitrocellulose. The nitrocellulose transfers were stained by the indirect immunoperoxidase method using mAb $VD2_1$.

blotting experiments with this antibody have shown that the 52,000 dalton subunit copurifies with the 175,000 and 170,000 dalton subunits of the nitrendipine receptor at all stages of the purification and is not a fragment of the larger subunits (Fig. 3). Immunoprecipitation experiments with ^{32}P-labeled nitrendipine receptor have demonstrated that the 170,000 and 52,000 dalton subunits are distinct phosphoproteins recognized by different monoclonal antibodies and that there exists a tight association between the 170,000 dalton subunit and the 52,000 dalton subunit of the receptor (Fig. 4).

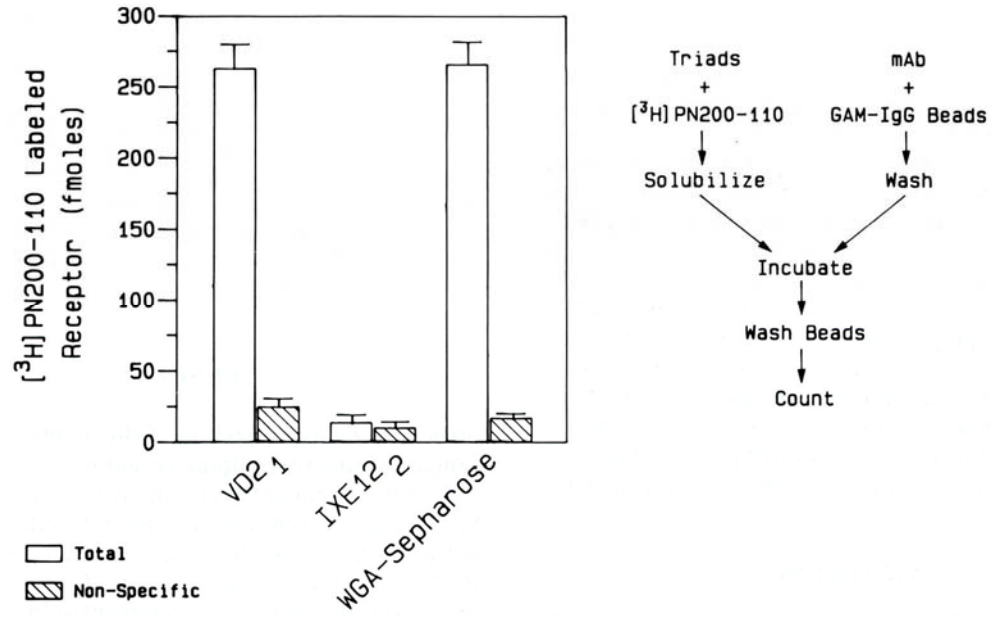

FIG. 2. Immunopreclpitation of the nitrendipine receptor with monoclonal antibody $VD2_1$. Hybridoma supernatants were tested for their ability to immunoprecipitate the radiolabeled receptor from digitonin-solubilized membranes (1). $IXE12_2$ is an unrelated antibody used as a control and WGA-Sepharose is used as a positive control. The nonspecifically labeled receptor was determined in the presence of 10 μm nitrendipine. The error bars represent the SEM from three independent repeats of the experiments.

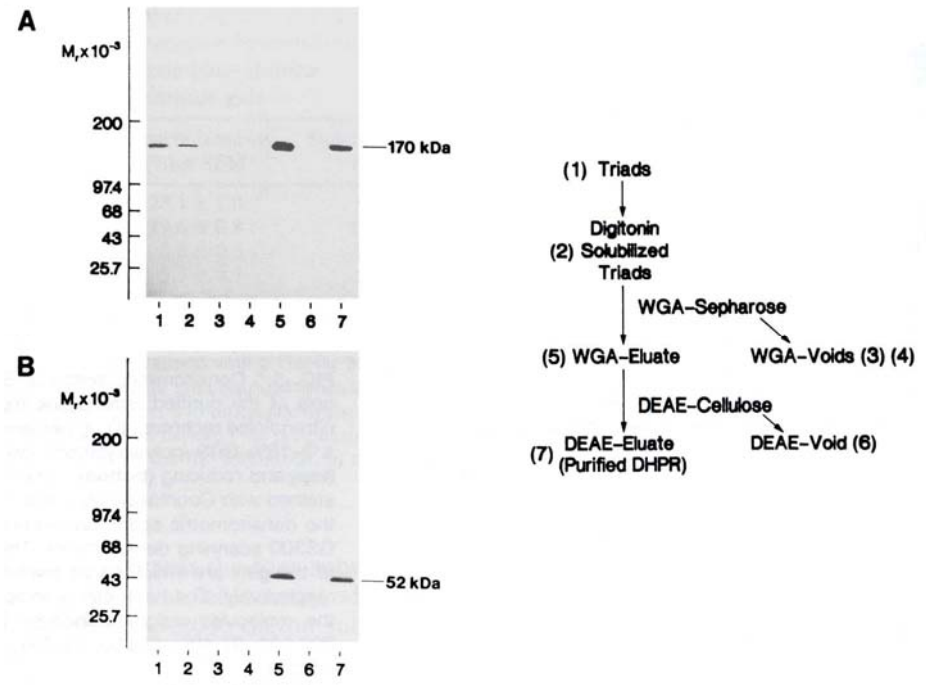

FIG. 3. Immunoblot staining of fractions from the purification of the nitrendipine receptor with monoclonal antibody $VD2_1$ (anti-52,000 dalton subunit). The various fractions from the purification of the nitrendipine receptor from triads were stained with mAb IIC12, anti-170 000 dalton protein **(A)** and mAb $VD2_1$, anti-52,000 dalton protein **(B)**. The samples on the transfers are membranes, 150 μg (lane 1); digitonin-solubilized membranes, 150 μg (lane 2); first void from WGA-Sepharose column, 150 μg (lane 3); second void from WGA-Sepharose column, 150 μg (lane 4); eluate from WGA-Sepharose, 10 μg (lane 5); void from DEAE-cellulose, 10 μg (lane 6); and peak fractions from DEAE-cellulose, 7.5 μg (lane 7).

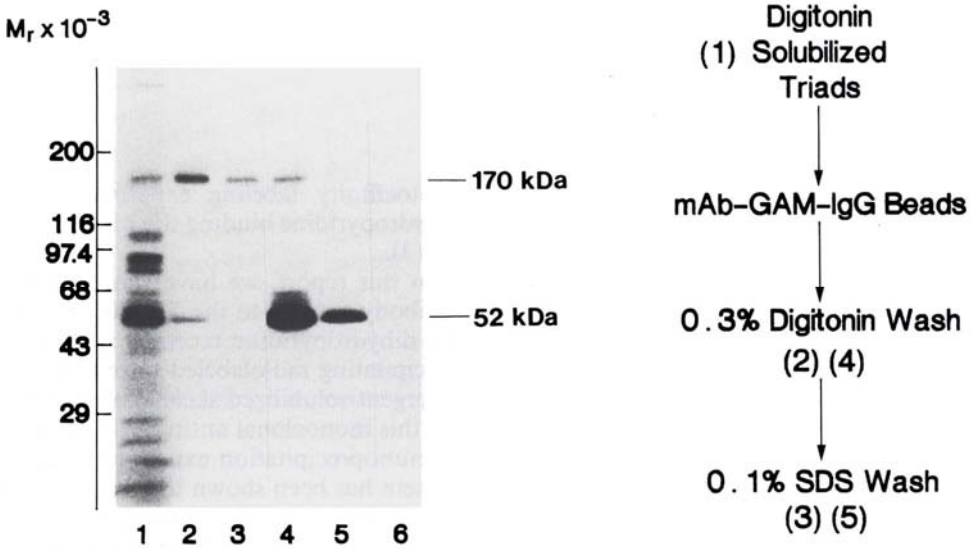

FIG. 4. Immunoprecipitation of the phosphorylated nitrendipine receptor. Isolated membranes were phosphorylated with 50 μM [γ-^{32}P]ATP and solubilized with 1% digitonln. Lane 1 contains 10 μg of digitonin-solubilized membranes. Digitonin-solubilized triads (100 μg) were incubated with mAb-GAM-IgG-Sepharose, mAb IIC12 (lanes 2 and 3), mAb VD2, (lanes 4 and 5), or GAM-lgG-Sepharose (lane 6) at 4°C for 4 h. The Sepharose beads were washed with 0.3% digitonin and 0.5*M* NaCl (lanes 2 and 4) or 0.1% SDS in 100 m*M* Tris-HCI (pH 7.5), 200 m*M* LiCI, and 20 m*M* NaF (lanes 3 and 5). Samples were subjected to SDS-PAGE on a 5-16% gradient gel under reducing conditions. The gel was stained with Coomassie blue, dried, and subjected to autoradiography.

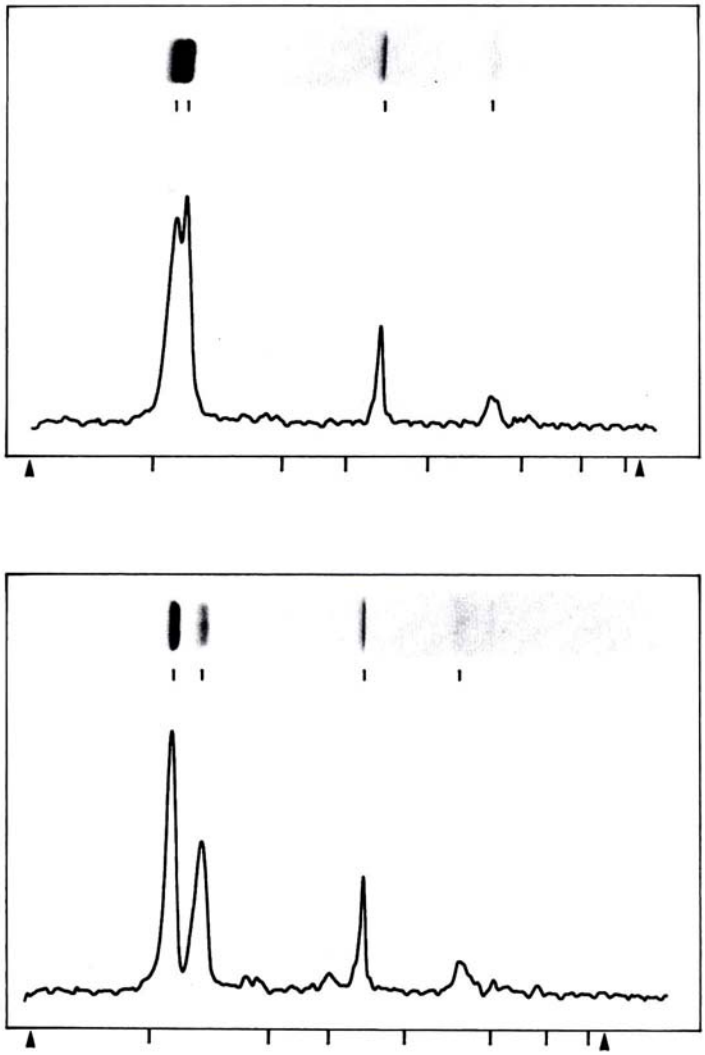

FIG. 5. Densitometric scans of SDS-polyacrylamide gels of the purified nitrendipine receptor. The purified nitrendipine receptor (10 μg per lane) was separated on a 5-16% SDS-polyacrylamide gel under nonreducing **(top)** and reducing **(bottom)** conditions. The gels were stained with Coomassie blue and, following destaining, the densitometric scans were obtained with a Hoefer GS300 scanning densitometer. The top and dye front of the gels are indicated by the left and right arrows, respectively. The hash marks indicate the positions of the molecular weight standards (from left to right): 200,000, 97,400, 43,000, 25,700, 18,400, and 14,300 daltons.

Determination of the subunit stoichiometry of the purified nitrendipine receptor

Densitometric scanning of Coomassie blue stained SDS-polyacrylamide gels of the purified nitrendipine receptor has shown that the 175,000, 170,000, 52,000, and 32,000 dalton subunits of the nitrendipine receptor exist in a 1:1:0.79:1 stoichiometric ratio (Fig. 5, Table 1). This ratio approaches 1:1:1:1 if the glycoprotem nature of the 175,000/150,000 dalton subunit is taken into consideration.

DISCUSSION

We have recently shown that the nitrendipine receptor purified from rabbit skeletal muscle contains four protein components of 175,000, 170,000, 52,000, and 32,000 daltons (1). The 175,000 dalton subunit is a WGA-positive glycoprotein whose apparent molecular weight on SDS-PAGE changes to 150,000 daltons upon reduction (1). The 170,000 and 52,000 dalton subunits have been shown to be phosphoproteins (2), and the 170,000 dalton subunit has been shown by photoaffinity labeling experiments to contain the dihydropyridine binding site of the nitrendipine receptor (3).

In this report, we have shown that a monoclonal antibody specific to the 52,000 dalton component of the dihydropyridine receptor is capable of immunoprecipitating radiolabeled nitrendipine receptor from detergent-solubilized skeletal muscle membranes. Using this monoclonal antibody in immunoblotting and immunoprecipitation experiments, the 52,000 dalton protein has been shown to be an integral and distinct subunit of the nitrendipine receptor. It has also been reported recently that this monoclonal antibody to the 52,000 dalton subunit is capable of activating the Ca^{2+} channel activity of the nitrendipine receptor reconstituted into planar lipid bilayers (10), further suggesting that the 52,000 dalton subunit does play a functional role in the regulation of the voltage-dependent Ca^{2+} channel.

Densitometric scanning of the purified nitrendipine receptor separated on SDS-PAGE and stained with

TABLE 1. *Determination of the subunit stoichiometry of the purified nitrendipine receptor by densitometric scanning of Coomassie blue-stained SDS-polyacrylamide gels*

Subunit	M_r	Relative intensity (%) ± SEM	Stoichiometric ratio
α_1	170,000	28.1 ± 1.0	1.00
α_2	175,000/150,000	19.6 ± 0.8	0.79
β	52,000	8.8 ± 3.3	1.03
γ	32,000	5.5 ± 0.3	1.04

The purified nitrendipine receptor from different preparations was subjected to SDS-PAGE on 5-16% gradient gels (10 μg per lane), stained with Coomassie blue and scanned with a Hoefer Model GS 300 scanning densitometer. The data are compiled from two scans of each of nine different preparations. No difference in the stoichiometric ratio of the subunits was seen between the preparations using CHAPS and those using digitonin for the sotubilization of the nitrendipine receptor.

n= 18.

Coomassie blue has shown that the four subunits of the receptor exist in a 1:1:1:1 stoichiometry, when the glycoprotein nature of the 175,000 dalton subunit is taken into consideration. Electron microscopy of the freeze-dried, rotary shadowed nitrendipine receptor purified from rabbit skeletal muscle revealed an ovoidal particle, 22 × 16 nm in dimension (11). The particle is large enough to contain all four subunits of the nitrendipine receptor and each particle is primarily composed of two components of similar size, separated by a small central gap (11).

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