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Immunochemical Analysis of Subunit Structures of 1,4-Dihydropyridine Receptors Associated with Voltage-Dependent Ca^{2+} Channels in Skeletal, Cardiac, and Smooth Muscles†

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ABSTRACT: Previous purification studies of the 1,4-dihydropyridine receptor associated with the calcium channel of rabbit skeletal muscle had shown that it is composed of a large glycoprotein of M_r 140 000–145 000 associated with a smaller component of M_r 32 000–34 000. Specific antisera have now been prepared against the larger component (anti-140 serum) and the smaller one (anti-32 serum). The specificity of these two antisera has been analyzed by immunoblot assays with microsomal preparations of rabbit skeletal muscle. Under disulfide-reducing conditions the anti-140 serum specifically labeled a polypeptide of M_r 140 000 while the anti-32 serum labeled three polypeptides of M_r 32 000, 29 000, and 26 000. Under nonreducing conditions both the anti-140 and the anti-32 sera specifically recognized a single large polypeptide of M_r 170 000. The same type of approach showed that the dihydropyridine receptor in cardiac and smooth muscles had a polypeptide composition similar to that found in skeletal muscle with a large polypeptide of M_r 170 000–176 000 made of two different chains of about M_r 140 000 and 34 000–32 000 associated by disulfide bridges.

Ca^{2+} channels exist mainly in excitable cells (Reuter, 1983; Tsien, 1983) such as muscle and nerve but also in secretory cells such as adrenal chromaffin cells (Baker & Knight, 1984) and in some nonexcitable cells such as sperm (Kazazoglou et al., 1985). Ca^{2+} movement through voltage-dependent Ca^{2+} channels is essential in excitation–contraction coupling in cardiac and smooth muscles. Ca^{2+} channel antagonists are currently used in the treatment of angina, supraventricular tachycardia, and hypertension and are potential drugs for other related pathologies (Janis & Triggle, 1984). 1,4-Dihydropyridines (DHP)[1] (e.g., nifedipine, nitrendipine, PN200-110)

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[1] Abbreviations: DHP, dihydropyridine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-(methoxycarbonyl)pyridine-3-carboxylate.

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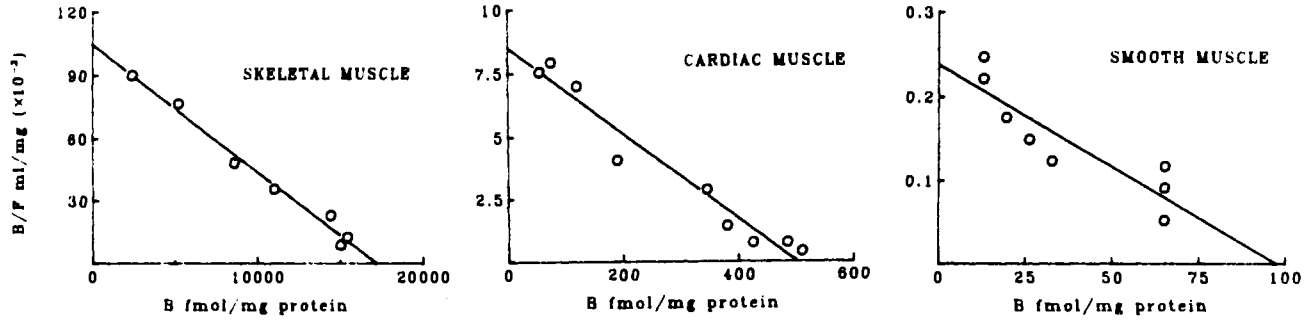

FIGURE 1: Typical Scatchard plots for the specific binding of (+)-[^{3}H]PN200-110 to skeletal, cardiac, and smooth muscle membranes. B/F is the ratio of specifically bound (+)-[^{3}H]PN200-110 to free (+)-[^{3}H]PN200-110. Experimental data in Scatchard plots are the mean of four determinations.

are Ca^{2+} channel antagonists acting on Ca^{2+} channels with high affinity and high selectivity (Sarmiento et al., 1983; Hof, 1984; Miller & Freedman, 1984; Janis et al., 1985; Schramm & Towart, 1985). Their receptor protein has now been purified (Borsotto et al., 1984, 1985; Curtis & Catterall, 1984) from the richest source of DHP receptors available, the T-tubule membranes of skeletal muscle (Fosset et al., 1983). Purification proved more difficult for cardiac (Rengasamy et al., 1985) or smooth muscle DHP receptors, which are pharmacologically more interesting than skeletal muscle DHP receptors. Affinity labeling experiments on cardiac and smooth muscle membranes have suggested a subunit structure (Venter et al., 1983; Horne et al., 1984; Campbell et al., 1984) different from that found for the skeletal muscle DHP receptor (Ferry et al., 1984; Galizzi et al., 1986).

We have raised antibodies against major polypeptide chains found for the skeletal muscle DHP receptor (Borsotto et al., 1985). These antibodies were then used in immunoblot experiments to compare the polypeptide composition of the DHP receptor, which presumably corresponds to the Ca^{2+} channel protein, in skeletal, cardiac, and smooth muscle cells.

Experimental Procedures

Membrane Preparation. Microsomal fractions from cardiac, smooth, and skeletal rabbit muscles were prepared as described previously for skeletal muscles by Fosset et al. (1983). Only ventricles were used for the cardiac microsome preparation. Smooth muscle microsomes were prepared from the colon portion of the intestine.

Immunoblot Assays. Samples of microsomes (80–100 μg) were denatured in 2% SDS, 9% glycerol, 75 mM Tris-HCl, pH 6.8, and 2.5% β-mercaptoethanol (disulfide-reducing conditions) or 8 mM iodoacetamide (nonreducing conditions) and loaded on a 4–14% linear polyacrylamide gradient according to Laemmli (1970). Proteins were transferred to nitrocellulose by using standard procedures (Gershoni & Palade, 1982). After protein transfer, nitrocellulose strips were incubated first with the saturating buffer (140 mM NaCl, 20 mM Tris-HCl, pH 7.4, 3% bovine serum albumin) for 2 h at room temperature and then overnight with different antisera. Blots were first washed 3 times with the washing buffer (140 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.2% Tween 20) and incubated for 2 h at room temperature with ^{125}I protein A [(2–3) × 10^6 cpm]. Blots were then washed 7 times with the washing buffer and autoradiographed on Kodak X-OMAT film with a Cronex Hi-plus intensifying screen (Du Pont). Staining of proteins was performed on separated strips in 25% isopropyl alcohol and 10% acetic acid containing 0.1% amido black (Gershoni & Palade, 1982).

Purification of the DHP Receptor Subunits from Rabbit Skeletal Muscle. Purification of the DHP receptor was carried out up to the wheat germ agglutinin step as described previously (Borsotto et al., 1985). The purified material [400 pmol of (+)-[^{3}H]PN200-110 binding sites/mg of protein] was run on polyacrylamide (4–14% linear gradient, 3-mm thickness) in the presence of SDS and 4% β-mercaptoethanol. The gels were briefly stained with Coomassie blue, and the portions containing the M_r 140 000 and 32 000 peptides were cut. The peptides were then electroeluted from the gel by using the method of Guevara et al. (1982).

Production of Guinea Pig Antisera. The purified peptides (50–80 μg) were injected first in complete Freund adjuvant into the footpads and in dorsal ganglions of young guinea pigs. Three weeks later, the same amount of material was injected in incomplete Freund adjuvant into the subscapular cavities and in dorsal ganglions. Finally, the guinea pigs were boosted again 1 week later by intraperitoneal injection of 50 μg of protein in 140 mM NaCl solution. Animals were bled 3 days after the last boost by cardiac punction and once a week during 1 month. Control antiserum was a mixture of sera obtained from six noninjected guinea pigs. Titers of different antisera were determined by serial dilutions on dot blot experiments with skeletal muscle microsomes (0.5 μg of protein) as antigen and ^{125}I protein A to reveal the presence of bound antibodies. A strong signal was still visible after a 10^6 dilution for the antiserum directed against the 32 000 protein, while only a weak signal was obtained at 10^5 dilution for the sera directed against the 140 000 protein. Protein A was iodinated to a specific activity of 74 TBq/mmol by the chloramine T method (Hunter & Greenwood, 1962).

Binding Assays. (+)-[^{3}H]PN200-110 (Amersham) binding to the three microsomal preparations was carried out at 4 °C, under dim light with GF/C filters according to standard procedures (Cognard et al., 1986).

Results and Discussion

Figure 1 shows Scatchard plots of binding experiments carried out with the potent tritiated (Cognard et al., 1986) Ca^{2+} channel inhibitor (+)-PN200-110 on microsomal fractions of the different tissues that will be used for immunoblotting experiments. Maximal binding capacities of 16 500, 500, and 100 fmol/mg of protein have been found for skeletal, cardiac, and smooth muscle (colon) membranes, respectively. K_d values are 0.15 nM for skeletal membranes, 0.06 nM for cardiac membranes, and 0.4 nM for smooth muscle membranes.

The difficulty encountered in the purification of DHP receptors from cardiac and smooth muscle cells comes (i) from the fact that they are much less abundant in membranes of these tissues than in skeletal muscle membranes (Figure 1) and (ii) from a higher lability of cardiac and smooth muscle DHP receptors after solubilization.

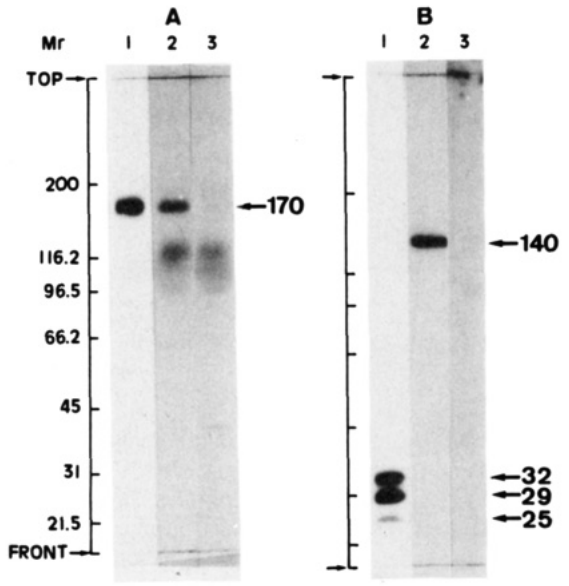

FIGURE 2: Immunoblots on rabbit skeletal muscle microsomes under nonreducing conditions (A) and under reducing conditions (B). Lane 1 with anti-32 serum (exposure time = 2 h), lane 2 with anti-140 serum (exposure time = 7 h), and lane 3 with control serum (exposure time = 7 h). All the antisera are used at a 10^3 dilution. Molecular weight markers are from Bio-Rad: myosin (200 000), β-galactosidase (116 500), phosphorylase B (96 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), and soybean trypsin inhibitor (21 500).

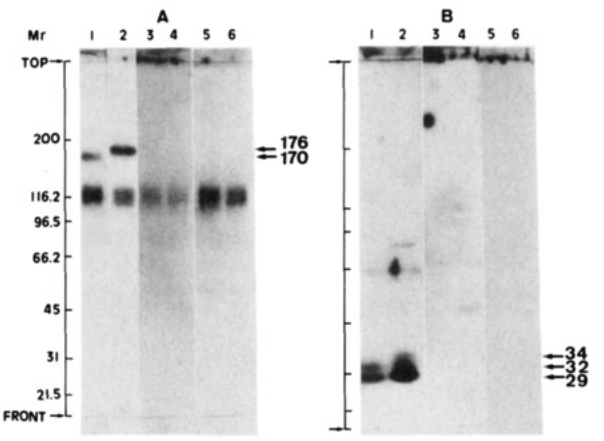

FIGURE 3: Immunoblots on rabbit cardiac and smooth muscle microsomes under nonreducing conditions (A) and under reducing conditions (B). Lanes 1, 3, and 5: smooth muscle with anti-32, anti-140, and control sera, respectively. Lanes 2, 4, and 6: cardiac muscle with anti-32, anti-140, and control sera, respectively. All sera are used at 10^3 dilution. Nonspecific labeling of a diffuse band of M_r 120 000 in nonreducing conditions was probably due to the presence in control and preimmune sera of immunoglobulin G against this peptide. Exposure time was 10 h for all lanes. Molecular weight markers are the same as in Figure 2.

The skeletal muscle DHP receptor has previously been shown to be made of two polypeptide chains at M_r 140 000 and 32 000–33 000 (Borsotto et al., 1984, 1985). Immunoblotting experiments performed on rabbit skeletal muscle membranes with the antiserum against the purified M_r 140 000 protein (anti-140 serum) showed that, under disulfide-reducing conditions (2.5% β-mercaptoethanol), this antiserum specifically labeled the large component of the Ca^{2+} channel protein of M_r 140 000 as expected (Figure 2B, lane 2). Under nonreducing conditions (Figure 2A, lane 2) the anti-140 serum specifically recognized a protein with a larger M_r of 170 000, suggesting that the small component of M_r 32 000–33 000, identified in purification studies (Borsotto et al., 1985), was probably covalently linked by disulfide bridges to the protein of M_r 140 000. In order to test this hypothesis, an antiserum directed against the purified M_r 32 000–33 000 component (anti-32 serum) was prepared, and its ability to react with the DHP receptor of skeletal muscle was studied in immunoblots. Figure 2B, lane 1, shows that one band revealed by anti-32 serum under disulfide-reducing conditions corresponded effectively to a low molecular weight of 32 000, but it also shows the presence of bands at M_r 29 000 and 25 000. Under nonreducing conditions the only peptide revealed by anti-32 serum had a M_r of 170 000 (Figure 1A, lane 1). Nonreducing conditions caused not only the complete disappearance of the band of M_r 32 000 but also the disappearance of the bands at M_r 29 000 and 25 000. This observation suggests that the two smaller polypeptides of M_r 25 000 and 29 000 are degradation products of the M_r 32 000 component. Other structural studies carried out in this laboratory that did not involve the use of antibodies confirmed this view (unpublished results). All these data taken together and obtained both with the anti-140 serum and the anti-32 serum indicate that the DHP receptor is indeed a protein of about M_r 170 000 made of two chains of M_r 140 000 and 32 000 covalently linked by disulfide bridge(s).

One important question was to know to what extent the molecular structure of the DHP receptor Ca^{2+} channel protein in cardiac and smooth rabbit muscles was similar to that found in skeletal muscle. In order to answer this question, immunoblotting experiments were carried out with microsomal fractions from cardiac and smooth muscles. Figure 3A (lanes 1, 2) shows that proteins of M_r 170 000 (smooth muscle) and M_r 176 000 (heart) were specifically labeled by the anti-32 serum under nonreducing conditions. Under disulfide-reducing conditions, after β-mercaptoethanol treatment, the anti-32 serum revealed smaller components of M_r 32 000 and 29 000 in the case of smooth muscle (Figure 3B, lane 1) and of M_r 34 000, 32 000, and 29 000 in the case of cardiac muscle (Figure 3B, lane 2). It is probable that the M_r 29 000 component in smooth muscle membranes is a degradation product of the M_r 32 000 peptide and that peptides at M_r 32 000 and 29 000 are degradation products of the M_r 34 000 peptide in cardiac muscle membranes. No specific labeling of peptide bands was observed with the anti-140 serum either in the presence or in the absence of the reducing agent (Figure 3A,B, lanes 3, 4). The presence in cardiac and smooth muscle membranes of the 170 000–176 000 protein under nonreducing conditions and of the 32 000–34 000 protein under reducing conditions clearly indicates, however, that a component of about M_r 140 000 exists in these tissues as in skeletal muscle. The lack of labeling of such a polypeptide component by the anti-140 serum could be due to the fact that antigenic determinants are different in skeletal muscle and in cardiac and smooth muscle DHP receptors. It could also be due to an insufficient titer of the anti-140 serum used. This second explanation seems more likely since the titer of the anti-140 serum was lower than the titer of the anti-32 serum and since the amount of DHP receptors measured with (+)-[^{3}H]-PN200-110 in heart and in smooth muscle was considerably lower than in skeletal muscle (Figure 1), making these receptors more difficult to detect in these tissues. Attempts to obtain more potent anti-140 sera were unsuccessful.

In conclusion, this work provides evidence that the DHP receptor that presumably corresponds to the Ca^{2+} channel protein of skeletal, cardiac, and smooth muscle has a very similar subunit composition in all these tissues comprising a large polypeptide of M_r 140 000 associated with a smaller polypeptide of M_r 32 000–34 000 by disulfide bridge(s). The

small subunit could be the same polypeptide that was previously photolabeled with [^{3}H]nitrendipine in canine cardiac membranes (Campbell et al., 1984).

The series of antibodies described in this paper can of course be very useful tools for future purifications of the cardiac and smooth muscle Ca^{2+} channel proteins and for the analysis of the distribution of these channels, as previously done for Na^+ channels (Ellisman & Levinson, 1982).

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Registry No. DHP, 3337-17-5; Ca, 7440-70-2.

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