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Monoclonal Antibodies That Coimmunoprecipitate the 1,4-Dihydropyridine and Phenylalkylamine Receptors and Reveal the Ca^{2+} Channel Structure†

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ABSTRACT: Monoclonal hybridoma cell lines secreting antibodies against the (+)-PN 200-110 and the (−)-demethoxyverapamil binding components of the voltage-dependent calcium channel from rabbit transverse-tubule membranes have been isolated. The specificity of these monoclonal antibodies was established by their ability to coimmunoprecipitate (+)-[^{3}H]PN 200-110 and (−)-[^{3}H]demethoxyverapamil receptors. Monoclonal antibodies described in this work cross-reacted with rat, mouse, chicken, and frog skeletal muscle Ca^{2+} channels but not with crayfish muscle Ca^{2+} channels. Cross-reactivity was also detected with membranes prepared from rabbit heart, brain, and intestinal smooth muscle. These antibodies were used in immunoprecipitation experiments with ^{125}I-labeled detergent [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and digitonin] solubilized membranes. They revealed a single immunoprecipitating component of molecular weight (M_r) 170 000 in nonreducing conditions. After disulfide bridge reduction the CHAPS-solubilized (+)-PN 200-110–(−)-demethoxyverapamil binding component gave rise to a large peptide of M_r 140 000 and to smaller polypeptides of M_r 30 000 and 26 000 whereas the digitonin-solubilized receptor appeared with subunits at M_r 170 000, 140 000, 30 000, and 26 000. All these results taken together are interpreted as showing that both the 1,4-dihydropyridine and the phenylalkylamine receptors are part of a single polypeptide chain of M_r 170 000.

Organic Ca^{2+} channel inhibitors have proved to be of great importance (Janis & Triggle, 1984; Miller & Freedman, 1984) in studies of the mechanism and of the molecular structure of the slow type of Ca^{2+} channel. The best known Ca^{2+} channel inhibitors include (i) 1,4-dihydropyridines such as nitrendipine and (+)-PN 200-110, (ii) verapamil-like compounds, and (iii) other types of molecules such as diltiazem and bepridil.

Skeletal muscle transverse tubule (T-tubule) membranes are the best source to study the properties of the 1,4-dihydropyridine-sensitive Ca^{2+} channel (Fosset et al., 1983; Ferry et al., 1984). The (+)-[^{3}H]PN 200-110 binding component of the channel protein has been detergent-solubilized and purified. However, different purification procedures in different laboratories have provided different evaluations of the subunit structure of the putative Ca^{2+} channel. It has been found in this laboratory that the 1,4-dihydropyridine receptor is a protein of M_r 170 000 assembled from a large subunit of M_r 140 000 and a smaller subunit of M_r 33 000–29 000, the two subunits being covalently linked by disulfide bridge(s) (Borsotto et al., 1984a, 1985; Schmid et al., 1986). It has also been found that the large subunit is the target of cAMP-dependent phosphorylation (Hosey et al., 1986). Conversely, it has been reported by another laboratory that the 1,4-dihydropyridine receptor is made by the assembly of three noncovalently linked polypeptides of M_r 160 000/130 000–50 000–33 000 (Curtis & Catterall, 1984), the peptide of M_r 50 000 being the one that is phosphorylated by a cAMP-dependent kinase (Curtis & Catterall, 1985). The first purpose of this work is to approach the problem of the subunit structure of the Ca^{2+} channel by a different route, which does not require purification procedures and uses monoclonal antibodies. The second purpose is to show that the two distinct receptors (Janis & Triggle, 1984) for 1,4-dihydropyridines and verapamil-like compounds are present in the same protein.

Experimental Procedures

Chemicals. (+)-[^{3}H]PN 200-110 and (−)-[^{3}H]demethoxyverapamil were from Amersham. *Staphylococcus aureus* V8 protease was from Sigma. Digitonin was from Serva. Methylcellulose was from Fluka and Pansorbin from Cal-

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biochem. Mouse immunoglobulin G (IgG) and rabbit anti-mouse IgG antibodies were from Miles and sheep anti-mouse IgG antibodies from Biosys. P_3-NSI-1-Ag4-1 myeloma cells were a gift of Dr. Van Obberghen. Protein A–Sepharose CL-4B was from Pharmacia. (+)-PN 200-110 was from Sandoz and (−)-D888 from Knoll.

Preparation of Membranes and of Detergent-Solubilized Extracts. T-Tubule membranes were prepared from rabbit skeletal muscle and assayed for receptors as previously described (Galizzi et al., 1984, 1986). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate- (CHAPS-) solubilized T-tubule membranes and CHAPS–wheat germ agglutinin (WGA) extract were prepared according to Borsotto et al. (1984b, 1985). Digitonin–WGA extract was prepared according to Curtis and Catterall (1984). Microsomes were isolated from mouse, chicken, and crayfish skeletal muscle and from rabbit heart and jejunum-ileum smooth muscle according to Galizzi et al. (1984). Rabbit brain synaptic membranes and rat and frog muscle T-tubule membranes were prepared as described (Fosset et al., 1983; Jones & Matus, 1974).

Production of Monoclonal Antibodies. A 6–7-week-old female BALB/c mouse received an intraperitoneal injection of rabbit T-tubule membranes (100 μg of protein) in Freund's complete adjuvant. Four weeks later, a second intraperitoneal immunization was performed. Three days later, the mouse was sacrificed. Spleen cells were fused with P^3-NSI-1-Ag4-1 myeloma cells according to Köhler and Milstein (1976). Hybridomas were selected for their ability to secrete relevant antibodies by an immunoprecipitation of (+)-[^{3}H]PN 200-110 bound to CHAPS-solubilized T-tubule membranes. Positive hybridomas were then cloned according to the methylcellulose technique (Davis et al., 1982). Ascites fluids were produced. The class and subclass of the heavy chains of the monoclonal antibodies were determined by the Ouchterlony immunodiffusion analysis.

Immunoprecipitation Assays. (*A*) *Precipitation of* (+)-[^{3}*H*]*PN 200-110 and* (−)-[^{3}*H*]*D888 Binding Activities.* Solubilized T-tubule membranes were incubated at 0 °C with 20 nM (+)-[^{3}H]PN 200-110 in the absence (total binding) or in the presence (nonspecific binding) at 1 μM unlabeled (+)-PN 200-110 for 10 min. Ten microliters of this mixture was added to a volume of 100 μL of culture supernatant or 100 μL of ascites dilutions. Control experiments were done with myeloma supernatant or culture medium containing 1–10 μg of mouse IgG instead of the culture supernatant (nonspecific binding). Incubations were continued for 1 h, after which time rabbit anti-mouse IgG antibodies (2 μg of specific anti-mouse IgG) were added (this step was omitted later on for monoclonal antibodies recognized by protein A). Two hours later, 10 μL of Pansorbin (*S. aureus* cell suspension) was added, and the solution was incubated for 15 min. (+)-[^{3}H]PN 200-110 associated with the receptor protein–antibody complex was separated from free (+)-[^{3}H]PN 200-110 by rapid filtration of a 100-μL aliquot from each sample on Whatman GF/C filters. The filters were washed 3 times with 100 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer at pH 7.5 and counted with Biofluor (New England Nuclear).

(+)-[^{3}H]PN 200-110 binding activity was precipitated from digitonin–WGA extract with monoclonal antibody 3007. Eight microliters of digitonin–WGA extract was incubated with 100 μL of ascites dilution. The immunoprecipitation assay was then processed as described for the CHAPS extract.

Immunoprecipitation of (−)-[^{3}H]D888 binding activity was carried out with ascites fluids previously characterized for their property of immunoprecipitating (+)-[^{3}H]PN 200-110 binding activity. Rabbit T-tubule membranes were prelabeled with (−)-[^{3}H]D888 before solubilization because it was difficult to demonstrate specific binding of this ^{3}H ligand to CHAPS-solubilized membranes. T-Tubule membranes (240 μg/mL) were incubated with 1 nM (−)-[^{3}H]D888 in the absence (total binding) or in the presence (nonspecific binding) of 1 μM (−)-D888 for 40 min at 0 °C. Then, solubilization was carried out as described previously and 10 μL of ascites dilution was added to a volume of 100 μL of supernatant of CHAPS-solubilized membrane. Immunoprecipitation was carried out as for the 1,4-dihydropyridine receptor.

(*B*) *Precipitation of Radioiodinated 1,4-Dihydropyridine Receptor.* Experiments were carried out at 0 °C. CHAPS-solubilized membranes and CHAPS– or digitonin–WGA extracts (20–50 μg of protein) were iodinated according to the lactoperoxidase method (Morrison & Bayse, 1970). Iodinated proteins were separated from free Na^{125}I by chromatography through a 5-mL Sephadex G-25 column equilibrated either in 0.1% CHAPS, 5% glycerol, 140 mM NaCl, and 20 mM Tris-HCl, pH 7.5 (buffer A) or in 0.1% digitonin, 185 mM KCl, 1.5 mM $CaCl_2$, 100 μM diltiazem, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)–Tris at pH 7.4 (buffer B). An aliquot of the iodinated proteins (10^6 cpm, 0.1–0.4 μg of protein) in a final volume of 100 μL of buffer A or buffer B was incubated for 2 h with 10 μL of a 2% (w/v) suspension of protein A–Sepharose CL-4B preincubated with either 10 μL of ascites fluid or 10 μg of mouse IgG as a control. The receptor–antibody complex was separated from other proteins by rapid filtration of samples on Sartorius 0.8-μm, SM 11104 filters. The filters were washed with 10 mL of buffer A without glycerol or buffer B and soaked with 120 μL of denaturing buffer containing 2% sodium dodecyl sulfate (SDS), 9% glycerol, 75 mM Tris-HCl, pH 6.8, and either 2.5% β-mercaptoethanol or 40 mM iodoacetamide and heated at 95 °C for 3 min. The denatured proteins were submitted to SDS–polyacrylamide gel electrophoresis performed on a 4–14% linear gradient according to Laemmli (1970). Gels were stained by Coomassie Blue, dried, and autoradiographed at −70 °C with Kodak XAR-5 film with intensifying screens.

(*C*) *Peptide Mapping.* Polypeptides immunoprecipitated from digitonin–WGA extract with antibody 3007 were subjected to SDS–polyacrylamide gel electrophoresis in β-mercaptoethanol, transferred by electroblotting to nitrocellulose (Gershoni et al., 1985), and submitted to iodination with chloramine T (Hunter & Greenwood, 1962). The iodinated proteins were subjected to one-dimensional peptide mapping (Cleveland et al., 1977).

Dot-Immunobinding Assays. These were carried out as described (Hawkes et al., 1982). The second antibody used was ^{125}I-labeled sheep anti-mouse IgG antibodies prepared as described (Pages et al., 1975).

Results and Discussion

Biochemical and electrophysiological experiments have previously shown that (+)-PN 200-110 is a very active 1,4-dihydropyridine calcium channel inhibitor of the mammalian skeletal muscle calcium channel (Cognard et al., 1986). Monoclonal antibodies were prepared by intraperitoneal injection in mice of purified rabbit T-tubule membranes.

The putative Ca^{2+} channel protein from T-tubule membranes has been previously detergent solubilized with CHAPS (Borsotto et al., 1984b, 1985) and purified with (+)-[^{3}H]PN 200-110. (+)-[^{3}H]PN 200-110 and its solubilized receptor form a stable complex with a half-life of dissociation of 34 h

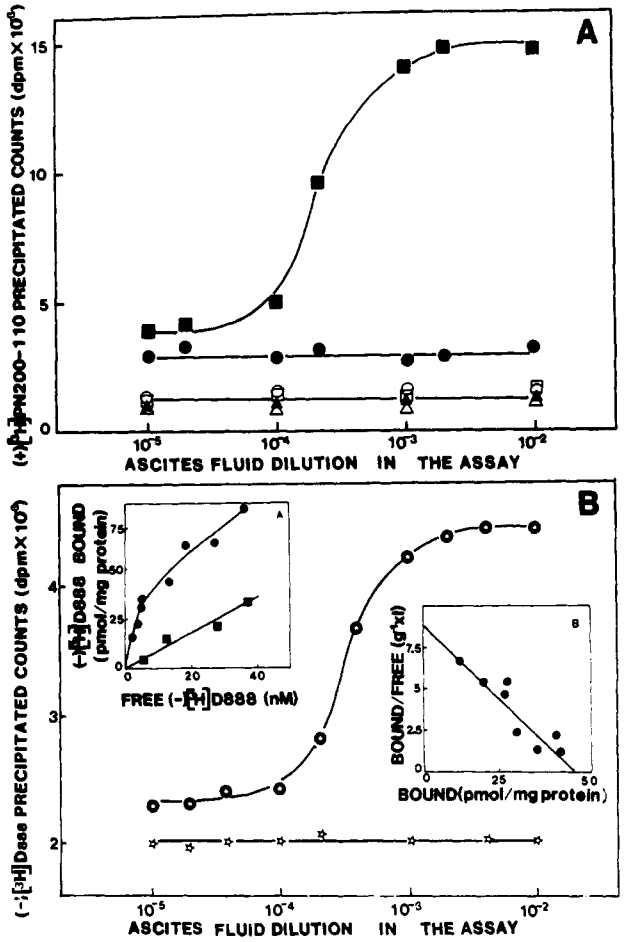

FIGURE 1: Immunoprecipitation of the (+)-[^{3}H]PN 200-110 and (−)-[^{3}H]D888 binding components by monoclonal antibody 3007 and equilibrium binding assay of (−)-[^{3}H]D888 to T-tubule membranes of CHAPS-solubilized form. Experiments were performed in duplicate. (A) The CHAPS-solubilized T-tubule membranes from rabbit skeletal muscle were incubated with (+)-[^{3}H]PN 200-110 at increasing concentrations of ascites fluid as described under Experimental Procedures. In this experiment, rabbit anti-mouse IgG antibodies were omitted. Immunoprecipitation with antibody 3007 in the absence (■) or in the presence (□) of 1 μM unlabeled (+)-PN 200-110. Nonrelevant ascites fluid in the absence (●) or in the presence (○) of 1 μM (+)-PN 200-110. Antibody 3007 without CHAPS-solubilized membranes in the absence (▲) or in the presence (△) of 1 μM (+)-PN 200-110. (B) Inset A: T-Tubule membranes at 20 μg/mL in 20 mM Hepes–NaOH buffer (pH 7.5 and 0 °C) were incubated for 60 min (time sufficient to attain equilibrium) with increasing concentrations of (−)-[^{3}H]D888 in the absence (●) or in the presence (■) of 1 μM (−)-D888. Each sample was then CHAPS solubilized as described under Experimental Procedures. Bound (−)-[^{3}H]D888 was separated from the free ligand by filtration (200-μL aliquots) through 2.8-mL Sephadex G-50 columns (Borsotto et al., 1984b). Inset B: Scatchard plot for the specific (−)-[^{3}H]D888 binding component. Main panel: T-Tubule membranes from rabbit skeletal muscle labeled with (−)-[^{3}H]D888 and CHAPS solubilized were incubated with increasing concentrations of ascites fluid as described under Experimental Procedures. Immunoprecipitation with antibody 3007 in the absence (✪) or in the presence (☆) of 1μM (−)-D888.

at 4 °C and an equilibrium dissociation constant K_D of 0.2–0.4 nM (Borsotto et al., 1984a,b). We took advantage of these properties of screening experiments with supernatants of hybridoma cultures in which we looked for supernatants able to precipitate the CHAPS-solubilized (+)-[^{3}H]PN 200-110–receptor complex. Hybridoma supernatants were considered to be positive when the amount of counts precipitated was at least twice the background value. Figure 1A shows a typical immunoprecipitation curve of the (+)-[^{3}H]PN 200-110 binding component as a function of ascites concentration for the 3007 hybridoma secreting monoclonal antibody. Precipitation of the nonspecific binding component was independent of the ascites concentration. The concentration of IgG necessary to give a half-maximum precipitation of the specific counts in Figure 1A was estimated to be 2–6 nM. Another type of purification using digitonin solubilization has been used by other authors (Curtis & Catterall, 1984). We have verified by using the same ascites that an identical type of immunoprecipitation curve was obtained for the digitonin extract of the (+)-PN 200-110 receptor (Figure 3A).

One important problem is to know whether monoclonal antibodies that immunoprecipitate the 1,4-dihydropyridine receptor also immunoprecipitate the phenylalkylamine (verapamil-like) receptor. It is known from binding studies that the receptors for those two categories of Ca^{2+} channel inhibitors are distinct and allosterically linked (Janis & Triggle, 1984). The first step was to analyze the properties of binding of (−)-[^{3}H]D888, a potent verapamil analogue (Galizzi et al., 1986), to solubilized T-tubule membranes.

Figure 1B, inset A, shows a typical equilibrium binding experiment of (−)-[^{3}H]D888 to CHAPS-solubilized skeletal muscle T-tubule membranes in the absence (total binding) and in the presence of 1 μM unlabeled (−)-D888. The Scatchard plot for the specific binding component (Figure 1B, inset B) was linear, indicating the presence of a single class of sites. The equilibrium dissociation constant was $K_D = 5.7 \pm 0.5$ nM. The affinity of (−)-[^{3}H]D888 for the solubilized receptor was only slightly altered by the solubilization procedure [$K_D = 1.5 \pm 0.5$ nM for the membrane-bound receptor (Galizzi et al., 1986)]. The maximum binding capacity was $B_{max} = 50 \pm 5$ pmol/mg of protein as for the membrane-bound receptor. Figure 1B, main panel, shows a typical immunoprecipitation curve of the solubilized (−)-[^{3}H]D888 binding component as a function of ascites fluid concentration for the 3007 hybridoma secreting monoclonal antibody. Precipitation of the nonspecific binding component was independent of the ascites concentration. The concentration of IgG necessary to give a half-maximum precipitation of the specific counts (Figure 1B) was estimated to be 2–6 nM, the same concentration as the one found for half-maximal precipitation of (+)-[^{3}H]PN 200-110 binding. We have also verified that prelabeling of the 1,4-dihydropyridine receptor with (+)-[^{3}H]PN 200-110 prior to CHAPS solubilization gave the same immunoprecipitation curve as that observed when labeling of the receptor with (+)-[^{3}H]PN 200-110 was posterior to solubilization.

Seven relevant clones were obtained out of a general screening of several thousand clones coming from different fusions between myeloma and spleen cells. All seven monoclonal antibodies had a high titer of 5000. The seven monoclonal antibodies were protein A sensitive. They belonged to the IgG class: IgG_3 (3007, 3106); IgG_1 (3329, 7447, 6322, 6332, 6308).

Dot-immunobinding assays against either native T-tubule or microsome membranes have indicated (not shown) that monoclonal antibodies against the rabbit skeletal muscle–1,4-dihydropyridine receptor cross-reacted with rat, mouse, chicken, and frog muscle membranes. No cross-reaction was found with crayfish muscle. Cross-reactivity was also found with membranes prepared from rabbit heart, brain, and intestinal smooth muscle.

In order to analyze the subunit structure of the immunoprecipitated protein(s) containing both the 1,4-dihydropyridine and the phenylalkylamine receptor, CHAPS-solubilized T-tubule and WGA extracts (Figure 2) and digitonin–WGA extract (Figure 3B) were iodinated and the ^{125}I-labeled

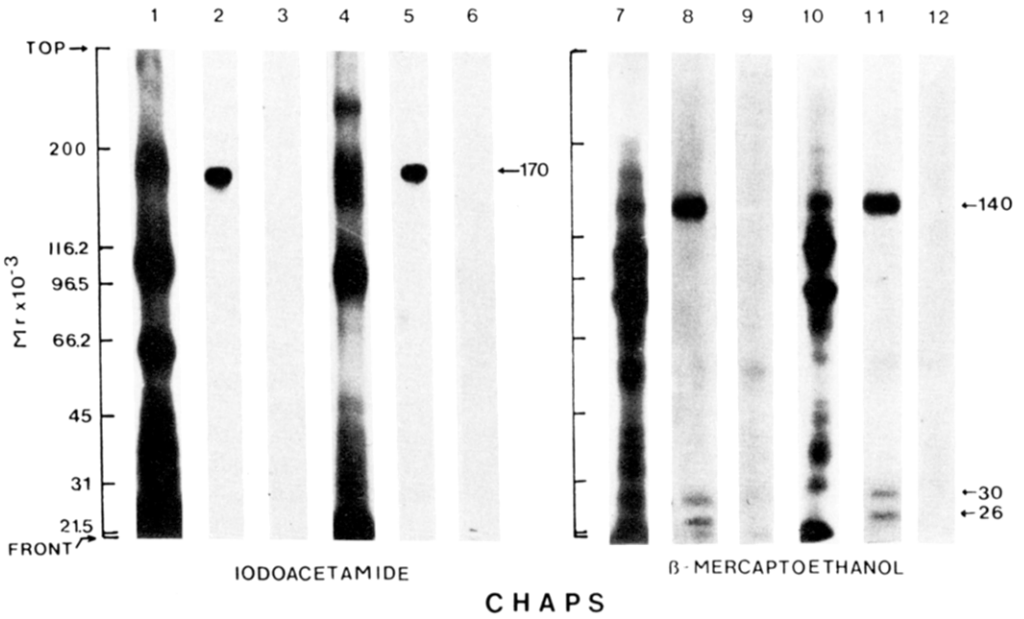

FIGURE 2: Immunoprecipitation by monoclonal antibody 3007 from the radioiodinated CHAPS-solubilized membranes and from the CHAPS–WGA extract of rabbit T-tubule membranes. These detergent extracts were iodinated and subjected to immunoprecipitation as described under Experimental Procedures. Autoradiogram: SDS–polyacrylamide gel electrophoreses were performed in the presence of iodoacetamide (lanes 1–6) or β-mercaptoethanol (lanes 7–12). Iodinated proteins from detergent-solubilized membranes (lanes 1 and 7; 10-μL aliquots from starting volume of 100 μL) or after immunoprecipitation by monoclonal antibody 3007 (lanes 2 and 8) and control mouse IgG (lanes 3 and 9). Iodinated proteins from WGA extract before (lanes 4 and 10) or after immunoprecipitation by monoclonal antibody 3007 (lanes 5 and 11) and control mouse IgG (lanes 6 and 12). Lanes 1, 4, 7, and 10 were exposed to the film for 5 h; the other lanes were exposed for 24 h. Molecular weight markers are presented at the left side of the figure.

preparations were submitted to immunoprecipitation by monoclonal antibodies. SDS–polyacrylamide gel electrophoresis were performed either in nondisulfide reducing conditions (iodoacetamide; Figure 2, lanes 1–6; Figure 3B, lanes 1–3) or in disulfide reducing conditions (β-mercaptoethanol; Figure 2, lanes 7–12; Figure 3B, lanes 4–6).

The typical monoclonal antibody 3007 was found to precipitate from both CHAPS and digitonin extracts a single polypeptide chain of apparent M_r 170 000 in nonreducing conditions (iodoacetamide; Figure 2, lanes 2 and 5; Figure 3B, lane 2).

In disulfide bridge reducing conditions, two different patterns were obtained, depending on the type of detergent used for solubilization: (i) polypeptides of apparent M_r 140 000, 30 000, and 26 000 were observed (Figure 2, lanes 8 and 11), when CHAPS was used for solubilization; (ii) when digitonin was used, polypeptides of M_r 170 000, 140 000, 30 000, and 26 000 were observed (Figure 3B, lane 5). Peptide maps of proteins of M_r 170 000 and 140 000 using *S. aureus* V8 protease showed that they were nearly indistinguishable (Figure 3C).

The seven selected monoclonal antibodies gave the same pattern of precipitation as antibody 3007 (not shown). Neither mouse IgG nor irrelevant monoclonal antibodies were able to specifically precipitate any identifiable component (Figure 2, lanes 3, 6, 9, and 12; Figure 3B, lanes 3 and 6).

Immunoprecipitation of the CHAPS-solubilized 1,4-dihydropyridine–phenylalkylamine receptor gave results similar to those of our previous purifications (Borsotto et al., 1984a, 1985). The intact receptor has a total M_r of 170 000; after disulfide reduction it is split into several pieces. One subunit, the large one, has a M_r of 140 000, identical with that found previously (Borsotto et al., 1984a, 1985; Schmid et al., 1986); the other ones have M_r's of 30 000 and 26 000 (Schmid et al., 1986). Presumably, the M_r 26 000 subunit is a degradation product of the M_r 30 000 subunit, as previously observed by us from purification studies (Schmid et al., 1986).

A subunit structure with a large chain of M_r 140 000 and a smaller chain of M_r 30 000 would be radically different from that found by others using a digitonin solubilization procedure (Curtis & Catterall, 1984) prior to purification. It is for that reason that monoclonal antibodies have also been used with the digitonin-solubilized material. It is clear from Figure 3B that, in these solubilization conditions too, the intact 1,4-dihydropyridine–phenylalkylamine receptor has a M_r of 170 000. Therefore, no differences are found between CHAPS and digitonin solubilizations for the nonreduced 1,4-dihydropyridine–phenylalkylamine receptor. However, in disulfide reducing conditions the gel pattern is different. The M_r 170 000 digitonin-solubilized receptor, unlike the CHAPS-solubilized receptor, is not completely converted into a M_r 140 000 protein. The easiest interpretation of this result is the following: (i) The 1,4-dihydropyridine–phenylalkylamine receptor is a single polypeptide chain of M_r 170 000 with internal disulfide bridges. (ii) This large polypeptide is vulnerable to proteolysis which is known, from Na^+ channel work (Casadei et al., 1984), to be very efficient in skeletal muscle. There is a "hot spot" for peptide cleavage capable of leading to the transformation of the M_r 170 000 unit in two pieces of M_r 140 000 and M_r 30 000, which remain covalently linked by disulfide bridges in nonreducing conditions. (iii) This cleavage is complete (in spite of the cocktail of protease inhibitors) in the CHAPS-solubilized material (M_r 170 000 $\rightarrow$ M_r 140 000 + 30 000 in reducing conditions) and only partial (M_r 170 000 $\rightarrow$ M_r 170 000 + 140 000 + 30 000) in the digitonin-solubilized material.

In conclusion, whatever the interpretation that one can make on differences of gel patterns in disulfide reducing conditions for the CHAPS- and digitonin-solubilized materials, this work clearly indicates that (i) the same protein is the receptor for both 1,4-dihydropyridines and phenylalkylamines, (ii) this protein has a M_r of 170 000, and (iii) other subunits of M_r 50 000 and 33 000 found by others (Curtis & Catterall, 1984) in nonreducing conditions are unlikely to belong to the re-

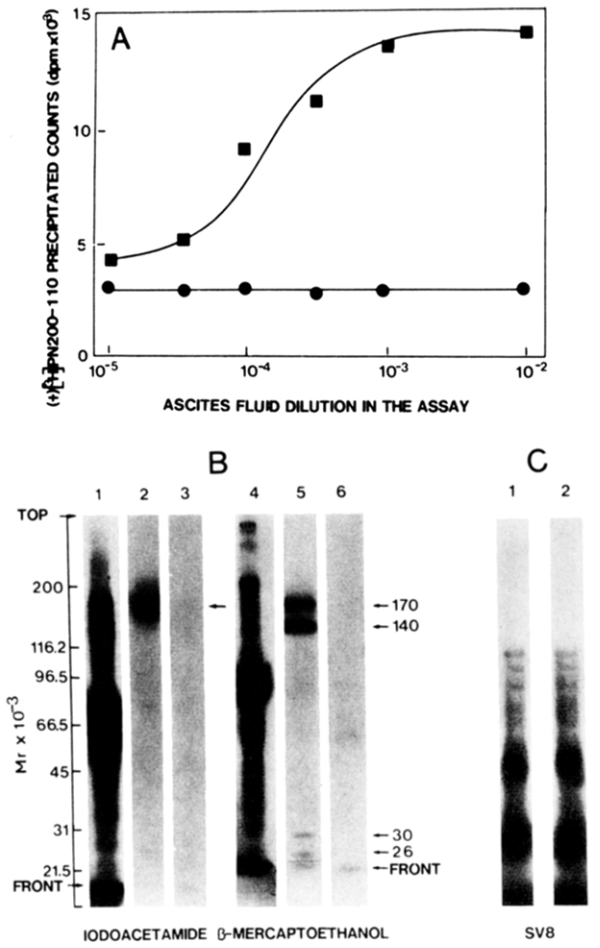

FIGURE 3: Immunoprecipitation by monoclonal antibody 3007 from digitonin–WGA extract and peptide maps. (A) Immunoprecipitation of the (+)-[^{3}H]PN 200-110 binding component. The digitonin–WGA extract labeled with (+)-[^{3}H]PN 200-110 was incubated at increasing concentrations of ascites fluids as described under Experimental Procedures. Immunoprecipitation with antibody 3007 in the absence (■) or in the presence (●) of 1 μM unlabeled (+)-PN 200-110. (B) Immunoprecipitation of the radioiodinated (+)-PN 200-110 receptor. The digitonin–WGA extract was iodinated and subjected to immunoprecipitation as described under Experimental Procedures. Autoradiogram: SDS–polyacrylamide gel electrophoreses were performed in the presence of iodoacetamide (lanes 1–3) or β-mercaptoethanol (lanes 4–6). Iodinated proteins from the digitonin–WGA extract (lanes 1 and 4) or after immunoprecipitation by monoclonal antibody 3007 (lanes 2 and 5) and control mouse IgG (lanes 3 and 6). (C) Peptide maps. The immunoprecipitated proteins obtained on nitrocellulose were iodinated and submitted to limited proteolytic digestion as described under Experimental Procedures. Autoradiogram: 140-kDa (lane 1) and 170-kDa (lane 2) peptides were submitted to limited proteolytic digestion with *S. aureus* V8 protease (0.3 μg of SV8, lanes 1 and 2).

ceptor. These monoclonal antibodies, because of their immunoprecipitating properties, will be very useful in future studies of the biosynthesis, integration, and histochemical localization of the Ca^{2+} channel protein at the surface membrane of excitable cells.

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