Is Ca^{2+} Antagonists Binding Protein from Cytosolic Fraction the Precursor of Alpha 1-Subunit of Ca^{2+} Channel?

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Abstract. The binding of Ca^{2+} antagonists to soluble proteins obtained by ammonium sulphate precipitation from cytosol fraction of rabbit skeletal muscles was studied. The K_D values for ^3H D-888 and ^3H PN 200-110 binding to soluble proteins were 21.3 ± 3.1 nmol . l^{-1} and 28.8 ± 8.9 nmol . l^{-1} respectively. Photoaffinity labelling of the soluble proteins with the arylazide 1,4-dihydropyridine probe ^3H azidopine resulted in labelling of the 85-95 K protein band as determined by SDS polyacrylamide gel electrophoresis. Partial purification of prelabelled soluble sample by gel filtration on Sephadex G-150 gave a more precise molecular weight of 90 ± 2.5K. Polyclonal antibodies prepared against Ca^{2+} channel complex from rabbit muscle T-tubules inhibited the ^3H PN 200-110 binding. Our results suggest that the soluble protein with Mr = $90K \pm 2.5K$ may be a precursor of the large subunit of the membrane bound L-type Ca^{2+} channel in rabbit skeletal muscle.

Key words: Ca^{2+} channel precursor — Ca^{2+} antagonist binding — Polyclonal antibodies

Introduction

Voltage-activated Ca^{2+} channels allow a rapid entry of calcium into cells in response to membrane depolarization (Froehner 1988). Three types of these channels have been described: L, T and N type (Nowycky et al. 1985). L-type of dihydropyridine sensitive Ca^{2+} channel has received considerable attention in

Abbreviations used: B_{max} — maximal binding capacity; K_D — dissociation constant; PMSF — phenylmethylsulphonyl fluoride; D-888 — desmethoxyverapamil; UV — ultraviolet; FA — complete Freund's adjuvans; SDS — sodiumdodecylsulphate, IgG — immunoglobulin G

the past several years. This channel has been studied in rabbit skeletal muscle (Curtis and Catterall 1984; Flockerzi et al. 1986), in skeletal muscle of guinea pigs (Glossman et al. 1985), human skeletal muscle (Križanová et al. 1988) and cardiac muscle (Cooper et al. 1987; Schneider and Hoffmann 1988). This channel type has been characterized both by using Ca^{2+} antagonists and by its purification and reconstitution into phospholipid vesicles (Curtis and Catterall 1986; Sieber et al. 1987). The identity of Ca^{2+} antagonist receptors isolated from various tissues was verified with polyclonal antibodies (Takahashi and Catterall 1987) and monoclonal antibodies (Norman et al. 1987; Malouf et al. 1987).

Soluble protein precursors of a number of transport systems have been well documented. The soluble protein precursors of Na^+ channel (Doyle et al. 1982), acetylcholine receptor (Carlin et al. 1986), (Na^+-K^+) ATPase (Tamkun and Fambrough 1986) and Na^+/Ca^{2+} exchanger (Juhászová et al. 1988) were identified.

The present work was aimed at investigating the existence of Ca^{2+} antagonist receptor in the soluble fraction of rabbit skeletal muscle.

Materials and Methods

Materials

^{3}H PN 200-110 (specific activity 3.15 TBq . mmol^{-1}), ^{3}H D-888 (specific activity 2.92 TBq . mmol^{-1}) and ^{3}H azidopine (specific activity 1.7 TBq . mmol^{-1}) were purchased from Amersham; D-600 was from Sigma, PN 200-110 was a generous gift of Dr. Lindenmann and Dr. Stähelin, Sandoz A. G.; scintillation cocktail SLT-41 and Bray's cocktail were from Spolana Neratovice; iodacetamide, PMSF, benzamidine and phenantroline were from Sigma; ^{125}I protein A was iodinated in our laboratory by the lactoperoxidase method according to Jørgensen et al. (1980); Sephadex G-150 was from Pharmacia; all other chemicals were from Serva (FRG) or Lachema (Czechoslovakia).

Buffers

Buffer A: 20 mmol . l^{-1} Tris-HCl pH 7.4, 0.3 mol . l^{-1} sucrose, 1 mmol . l^{-1} $CaCl_2$, 1 mmol . l^{-1} iodacetamide, 1 mmol . l^{-1} PMSF, 1 mmol . l^{-1} benzamidine and 1 mmol . l^{-1} phenantroline; Buffer X: 20 mmol . l^{-1} Hepes-NaOH pH 7.4, 1 mmol . l^{-1} $CaCl_2$; Buffer Y: 20 mmol . l^{-1} Tris-HCl pH 8.0, 5 mmol . l^{-1} EGTA; Buffer TBS: 50 mmol . l^{-1} Tris-HCl pH 8.0, 150 mmol . l^{-1} NaCl; Buffer C: 20 mmol . l^{-1} Tris-HCl pH 7.4.

Preparation of membranes and proteins

T-tubule membranes were prepared according to Rosemblatt et al. (1981). Rabbit muscle tissue was cut into small pieces and homogenized in buffer A in an ETA 0010 knife homogenizer. The homogenate was centrifuged at 3000×g 10 min. The obtained sediment was rehomogenized and

centrifuged at 11,000x*g* 20 min. To the supernatant KCl was added to a final concentration of 0.5 mol.l^{-1}. The solution was mixed for 1 hour at 4°C and subsequently centrifuged on a Beckman ultracentrifuge L-8-80 at 150,000x*g* for 60 min. The sediment was resuspended in a small volume of Tris-HCl buffer, pH 7.4 and either used for further studies (as crude membrane fraction) or separated on discontinual sucrose gradients (25, 35, 40 and 50 %) at 85,000x*g* for 16 hours. The T-tubule membrane fraction was collected from the limit between 0—25 % sucrose.

To the supernatant ammonium sulphate was added to reach 50 % saturation, and the solution was mixed for 1 hour at 4°C. After centrifugation at 50,000x*g* for 30 min the sediment was redissolved in a small volume of buffer X and was denoted the soluble fraction. To check possible admixture of membranes, Na^+-K^+ ATPase activity was determined by the method of Ziegelhöffer et al. (1983) in the soluble fraction. Proteins were estimated according to Lowry et al. (1951).

Incorporation of soluble proteins into azolectin vesicles

5 mg of azolectin was dissolved in aceton and evaporated under nitrogen. 1 mg of soluble proteins in buffer X was added to azolectin and the mixture was shaked 30 min at 4°C. The obtained proteoliposomes were sonicated 5 min on ice and used for binding studies.

Binding assays with ^3H PN 200–110

Binding was carried out in buffer X in the presence of 1—5 nmol.l^{-1} ^3H PN 200—110. PN 200–110 (0—2 μmol.l^{-1}) was added to estimate K_D and B_{max}. Samples containing membrane or soluble proteins, ^3H PN 200–110 and unlabelled PN 200–110 in a final volume of 0.5 ml of buffer X were incubated for 60 min at 20°C. Because of the light sensitivity of PN 200–110, all steps were carried out at yellow light. After the incubation 50 μl albumin (5mg. ml^{-1}) was added. After another 5 min the samples were filtered through Whatman GF/B filters and washed twice with 4 ml of buffer X. The radioactivity of the samples was measured in scintillation cocktail SLT-41 on Rackbeta LKB equipment. B_{max} and K_D were estimated from Scatchard plots (Scatchard 1949).

Binding assays with ^3H D-888

The binding assays were done in buffer Y in the presence of 0.5—1 nmol.l^{-1} ^3H D-888. The concentration of unlabelled D-600 varied between 0 and 2 μmol.l^{-1}. The samples were incubated for 60 min at 20°C, then 50 μl albumin (5 mg.ml^{-1}) was added and after additional 5 min the samples were filtered through Whatman GF/B filters and washed twice with 4 ml of buffer Y each. Scintillation cocktail SLT-41 was added and the radioactivity of each sample was measured. B_{max} and K_D were estimated from Scatchard plots (Scatchard 1949).

Photoaffinity labelling of Ca^{2+} antagonists binding proteins

Photoaffinity labelling was done by the method of Ferry et al. (1984) in a slight modification. The total volume of the labelling mixture was 0.5 ml. Both the T-tubule membrane fraction and the soluble fraction were incubated with 2 nmol.l^{-1} ^3H azidopine each in the presence or absence of 1—10 μmol.l^{-1} PN 200–110. After 60 min incubation at 20°C the fractions were put on ice and irradiated for 7 min with a UV lamp (UVP Inc. San Gabriel California, USA) from a distance of 10 cm. To the labelled samples the sample buffer was added and the incubation continued at 90°C for 5 min. The molecular weights of the labelled proteins were measured electrophoretically under nonreducing conditions according to Laemli (1970) in 8% slab gels. The low molecular weight calibration kit from Pharmacia was used as the molecular weight marker. After staining the gels

were cut into 3 mm (soluble fraction) or 5 mm (T-tubule membrane fraction) stripes. The stripes were solubilized overnight in Bray's scintillation cocktail (Ferry et al. 1984) and the radioactivity of each strip was measured.

Preparation of polyclonal antibodies

Polyclonal antibodies against T-tubule membranes were produced in BALB/c mice aged 6—8 weeks. The mice were immunized with subcutaneous injections of rabbit T-tubule membranes with or without complete Freund's adjuvans, and boosted by three additional injections. Complete immunization took 1 month without FA and three months with FA. The animals were decapitated 5—7 days after the last immunization and the hyperimmune serum was obtained. Preimmune serum was obtained from noninjected mice. Polyclonal antibodies were tested by immunoblot analysis. Membrane proteins were transfered from SDS-polyacrylamide gels on nitrocellulose membranes (HYBOND C). The nitrocellulose membranes were then briefly washed in TBS solution and TBS supplemented with calf fetal serum (TBSc), and incubated with hyperimmune serum in TBSc for 2 hours at room temperature. After washing with TBS and TBSc, ^{125}I labelled protein A was added and the mixture was incubated for 60 min at room temperature. All steps were performed under gentle shaking. The membranes were washed thrice with TBS and TBSc and subsequently autoradiographed for 24 hours.

Effect of polyclonal antibodies on ^{3}H PN 200-110 binding

For the testing of the effect of polyclonal antibodies against T-tubule membranes on ^{3}H PN 200 – 110 binding, crude membranes or soluble fraction, each containing 50—100 μg proteins, were used. To these proteins 1 to 5 nmol.l^{-1} ^{3}H PN 200 – 110 and 20 μl of either preimmune or hyperimmune serum or buffer X were added. Samples with unlabelled PN 200 – 110 served as controls. The samples were incubated for 1 hour at 20 °C in a final volume of 0.5 ml. Subsequently 50 μl albumin (5 mg . ml^{-1}) was added and after additional 5 min the samples were passed through GF/B filters and washed twice with buffer X. After the addition of SLT-41 scintillation cocktail the radioactivity of the filters was determined on Rackbeta LKB equipment.

Partial purification of Ca^{2+} antagonists binding protein in soluble fraction by gel chromatography

The soluble fraction was prelabelled with azidopine as described above, and passed through Sephadex G-150 column (50 × 1 cm). The radioactivity peak was collected, lyophilized and the protein profile was determined electrophoretically on SDS 8% slab gels under nonreducing conditions.

Results

Specific binding of Ca^{2+} antagonists

To check whether specific binding occurs in the soluble fraction the specific binding of ^{3}H D-888 was determined in proteins salted out with 50% ammonium sulphate saturation.

Table 1. K_D and B_{max} values estimated from Scatchard plots.

A — Binding of ^{3}H D-888

fraction	K_D (nmol.l^{-1})	B_{max} (pmol.mg^{-1} prot)
soluble fraction	21.3 ± 3.1	4.5 ± 1.3
T-tubule membranes	13.9 ± 2.6	125.4 ± 6.6
crude membrane fraction	8.6 ± 3.2	5.6 ± 1.3

B — Binding of ^{3}H PN 200–110

fraction	K_D (nmol.l^{-1})	B_{max} (pmol.mg^{-1} prot)
soluble fraction	28.8 ± 8.9	3.4 ± 0.9
T-tubule membranes	15.6 ± 3.8	281.7 ± 7.7
crude membrane fraction	23.6 ± 3.6	46.5 ± 0.2

Mean values ± SEM of at least three measurements each run in three parallel experiments are shown.

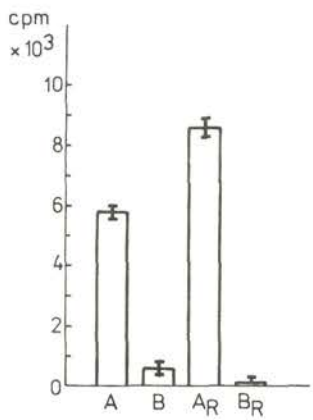

Fig. 1. The specific binding of ^{3}H D-888 to soluble fractions from rabbit skeletal muscle. A — soluble proteins were precipiteted with ammonium sulphate to 50%; *B* — soluble proteins were precipitated from 50 to 100%; A_R — fraction A after reconstitution to phospholipid vesicles; B_R — fraction B after reconstitution to phospholipid vesicles

The results summarized in Fig. 1 showed that ^{3}H D-888 binds specifically to the proteins from soluble fraction (column A). This binding could be enhanced by incorporation of the soluble proteins into phospholipid vesicles (column A_R). When the supernatant after 50% ammonium sulphate precipitation was saturated with ammonium sulphate to 100%, there was practically no specific

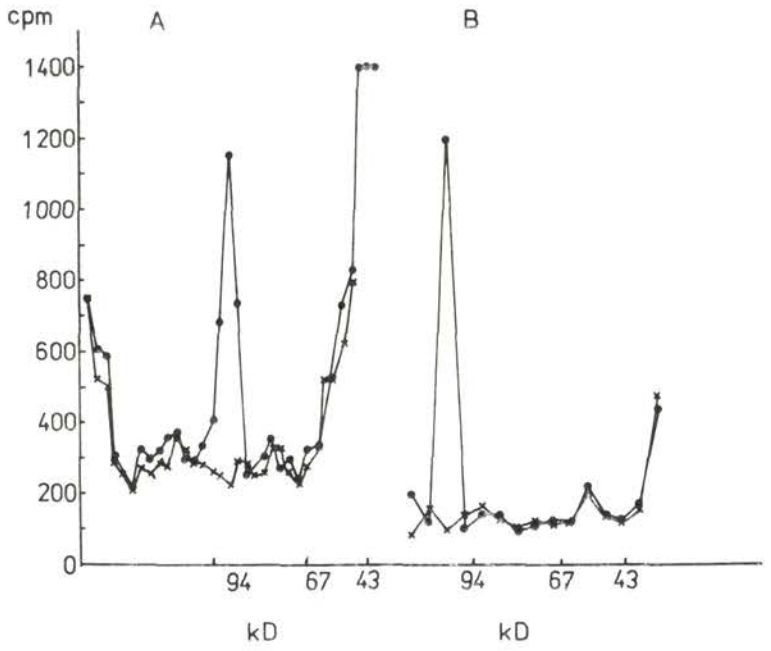

Fig. 2. Molecular weight determination of the ^{3}H azidopine binding protein; *A* — soluble fraction, *B* — T-tubule membrane fraction. For details of the experiment see Materials and Methods. —x—x—x— — nonspecific binding, —●—●—●— — total binding. The result shown are averages of three experiments.

binding of ^{3}H D-888 either to free proteins (column B) or to proteins incorporated to phospholipid vesicles (column B_R). Prompted by the above results, Scatchard analysis was performed on fraction A with ^{3}H D-888 and ^{3}H PN 200–110. To allow comparison, the crude membrane fraction and the T-tubule membrane fraction were tested identically. Values of B_{max} and K_D for PN 200–110 and D-888 are shown in Table 1. B_{max} for D-888 binding to soluble fraction is close to that for the crude membrane fraction; B_{max} for D-888 binding to T-tubule membranes is larger because of both higher concentrations of Ca^{2+} antagonist receptors and less secondary proteins present in this fraction as compared to the soluble fraction. The slightly higher values of K_D for both D-888 and PN 200–110 binding to soluble fraction may indicate that this fraction has high affinity domains of Ca^{2+} antagonist receptor a little modified from those of T-tubule membranes.

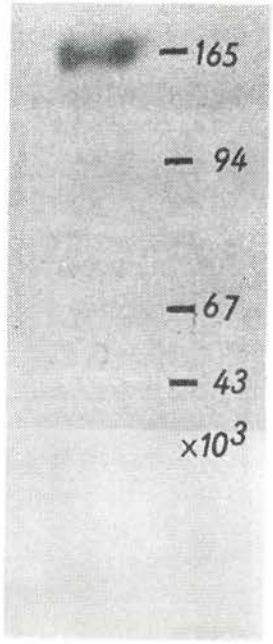

Fig. 3. Autoradiogram of an immunoblot of polyclonal antibodies to membrane fraction. Antibodies against Ca^{2+} channel bind to a 165K protein.

The photoaffinity labelling of Ca^{2+} antagonists binding protein

After irradiation, arylazide 1,4-dihydropyridine ^{3}H azidopine binds covalently to both soluble fraction and the T-tubule membrane fraction (Fig. 2). The molecular weights of these proteins were estimated electrophoretically.
In the soluble fraction azidopine labelled a protein with molecular weight of 85—95 K, while in T-tubule membranes the respective protein had a molecular weight of 150—170 K.

Effect of polyclonal antibodies on ^{3}H PN 200 – 110 binding

The quality of the hyperimmune serum was tested by immunoblot. In crude membrane fraction the polyclonal antibodies against Ca^{2+} antagonist receptor bound specifically to a protein with Mr of 165 K (Fig. 3). This protein is assumed to be the large subunit of the Ca^{2+} antagonist receptor. Owing to this, the effect of the hyperimmune serum on the binding of ^{3}H PN 200 – 110 to the soluble fraction and the crude membrane fraction was tested. Because of the nonspecific interaction of ^{3}H PN 200 – 110 bindig with serum, the preimmune

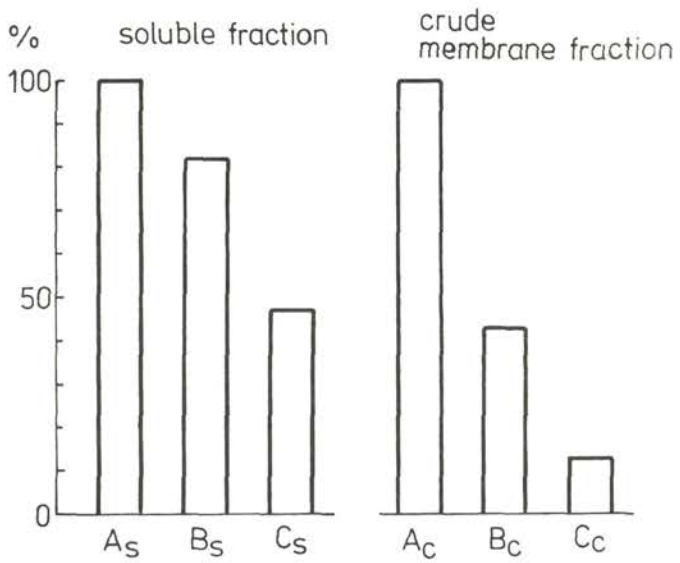

Fig. 4. The effect of polyclonal antibodies on ^{3}H PN 200 – 110 binding. Binding of ^{3}H PN 200 – 110 to soluble fraction (A_s) and/or crude membrane fraction (A_c) was taken as 100%. Preimmune serum reduced ^{3}H PN 200 – 110 binding to the soluble fraction (B_s) to 82.5%, while the corresponding value for hyperimmune serum (C_s) was 47.2% For crude membrane fraction the reduction was 43% and 13% for preimmune (B_c) and hyperimmune (C_c) serum, respectively. The difference between preimmune and hyperimmune serum effects is probably due to polyclonal antibodies. This figure shows typical results from 3 experiments.

serum served as a control. Typical results from three experiments are shown in Fig. 4. In the soluble fraction preimmune serum reduced the ^{3}H PN 200 – 110 binding to 82.5% and the hyperimmune serum to 47.2% of the control values. The difference between ^{3}H PN 200 – 110 binding in the presence of preimmune serum and hyperimmune serum is probably caused by polyclonal antibodies against Ca^{2+} antagonist receptor. The same experiment was done with crude membrane fraction as a control. Preimmune serum lowered the ^{3}H PN 200 – 110 binding the ^{3}H PN 200 – 110 binding to 43% and hyperimmune serum to 13% of the control values.

Partial purification of the Ca^{2+} antagonists binding protein from cytosol

After prelabelling of soluble fraction with azidopine and irradiation with UV light for 10 min, the sample was passed through Sephadex G-150 column. The

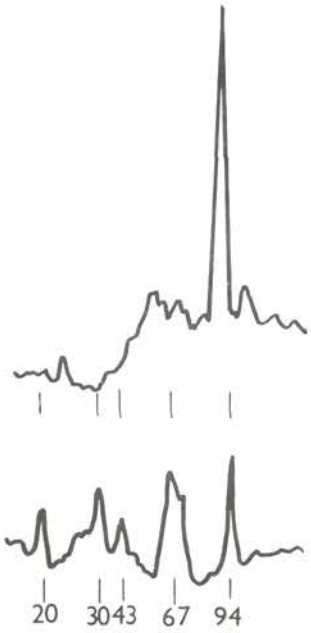

Fig. 5. Densitogram from the sample partially purified on Sephadex G-150 column (upper curve). The concentration of 90K ± 2.5K protein, to which the ^3H labelled azidopine bound, was much higher than in unpurified sample. The lower curve correspond with the low molecular weight calibration kit.

fractions from radioactivity peak were collected and determined on SDS electrophoresis after lyophilization. The electrophoresis showed that radioactivity was predominantly bound to a major protein with Mr 90,000 ± 2500 (Fig. 5).

Discussion

Ca^{2+} antagonists are drugs of considerable clinical importance; they inhibit one type of Ca^{2+} channel, the L-type. The calcium channel and/or the Ca^{2+} antagonists receptor are able to bind Ca^{2+} antagonists specifically, though some nonspecifical binding sites were also observed in the membrane fraction. There was no evidence for the presence of Ca^{2+} antagonist binding protein in the cytosolic fraction. Possible contamination of the soluble fraction with membrane fragments was tested by determining the activity of $Na^+ - K^+$ ATPase in this sample. Since no $Na^+ - K^+$ ATPase activity was present it may be assumed that the fraction was free of membranes. In our experiments Ca^{2+} antagonists

^{3}H D-888 and ^{3}H PN 200 – 110 bound to the soluble protein fraction with K_D 21.3 ± 3.1 nmol . l^{-1} and 28.8 ± 8.9 nmol . l^{-1}, respectively. These K_D values are similar to those obtained for membrane fractions with the same Ca^{2+} antagonists (Table 1). Binding of Ca^{2+} antagonits to the soluble fraction might indicate that in this fraction there are proteins with similar high affinity domains for binding Ca^{2+} antagonists as in performed membranes.

The molecular weight of the major subunit of the membrane Ca^{2+} antagonist receptor was estimated with ^{3}H azidopine (Ferry et al. 1986; Flockerzi et al. 1986; Ferry et al. 1987). Only this protein with Mr 150—170K is able to specifically bind Ca^{2+} antagonists as could be shown also in our experiments. Molecular weights of the soluble Ca^{2+} antagonists binding proteins from rabbit skeletal muscle, estimated with ^{3}H azidopine, was 85—95K, i.e. approximately half of the value obtained for the ^{3}H azidopine binding protein from membrane fraction. When partially purified on Sephadex G-150, the protein showed a Mr of 90 ± 2.5K. Nevertheless it may be assumed that the protein with this Mr, which was found to bind Ca^{2+} antagonists, is the precursor of the large subunit of the Ca^{2+} antagonist receptor. This contrasts with the report by Haga et al. (1984), who found a protein with Mr less than 30K, which is responsible for the moving activity in Paramecium. This discrepancy might be due to differences in experimental material, which is phylogenetically rather different.

The relation between Ca^{2+} antagonist receptor and 90 ± 2.5K soluble protein was also checked with the use of polyclonal antibodies against membrane bound Ca^{2+} antagonist receptor (Fig. 4) reduced ^{3}H PN 200 – 110 binding to both the membrane and the soluble fraction. The partial inhibitory effect of preimmune serum could be caused by class G immunoglobulins (IgG). These IgG contain the binding domains which are able to bind nonspecifically to a wide range of mammalian proteins. The difference between the effects of preimmune and hyperimmune serum can be ascribed to specificity of the polyclonal antibodies against the Ca^{2+} antagonist receptor. Our results agree with those reported by Repke and Liebmann (1987), who have shown that myasthenic serum which contains antibodies against acetylcholine receptor reduced the specific binding of ^{125}I alpha-bungarotoxin to nicotinic receptor. Similar observations made in our experiments with both the membrane and the soluble fraction using polyclonal antibodies support our assumption concerning the relation between Ca^{2+} antagonist receptor and 90K ± 2.5K soluble protein. This protein could be the precursor of the Ca^{2+} antagonist receptor. The existence of a soluble protein precursor for membrane bound Na^+ channel was previously well documented in cytosolic fractions of dog heart and rat brain (Doyle et al. 1982; Malysheva et al. 1984; Schmidt et al. 1985). The proteins after isolation and reconstitution into liposomes were able to bind ^{3}H tetrodotoxin and to transport ^{22}Na. Reports from other laboratories (Carlin et al.

1986; Tamkun and Fambrough 1986) have also shown that the protein precursor of membrane bound alpha subunit of acetylcholine receptor with a high affinity to alpha bungarotoxin is present in the cytosolic fraction of mouse muscle cell line BC3H-1. In addition, the analysis of protein synthesis of alpha and beta subunits of normal (Na^+-K^+) ATPase during or immediately after polypeptide insertion into the plasma membrane has revealed that only 45 to 70% of the newly formed proteins were membrane bound. Juhászová et al. (1988) could reconstitute soluble Na^+/Ca^{2+} exchanger from crayfish striated muscle into azolectin proteoliposomes.

Johnson et al. (1988) digested the ^3H PN photolabelled calcium channel with V-8 protease. They have found, that during the initial stages of these digestions with low concentrations of protease (5 μg), the 160K protein was completely degraded into three photolabelled fragments of 85K, 33K and 28K. Digests at higher protease concentrations (10 μg) resulted in a small decrease in the 85K form and a corresponding increase of 28K form. The question rised up, whether the 85K digest from 160K subunit and the soluble protein with 90K $\pm$ 2.5K are related proteins or not.

It is highly probable that the soluble 90K $\pm$ 2.5K protein is the precursor of the alpha 1 subunit of Ca^{2+} channel in rabbit skeletal muscle. However, further studies with purified and reconstituted protein are needed for definitive conclusions to be drawn.

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