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PURIFICATION OF THE CARDIAC 1,4-DIHYDROPYRIDINE RECEPTOR/ CALCIUM CHANNEL COMPLEX

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SUMMARY. Chick heart membranes were labelled with [^{3}H]PN 200-110, a 1,4-dihydropyridine calcium channel antagonist. The [^{3}H]PN 200-110 receptors were solubilized with digitonin and purified 600-fold using several chromatographic systems and sucrose density gradient centrifugation. Analyses by gel electrophoresis revealed that the final product was enriched in two peptides with molecular weights of 60,000 and 54,000, and another of 34,000.

The opposing effects of norepinephrine and acetylcholine on the contractile force of the heart are believed to result from their respective abilities to induce phosphorylation and dephosphorylation of the voltage-sensitive calcium channel or a regulatory protein (1). The exact biochemical events involved in the regulation of the calcium channel remain unknown largely because the proteins that comprise, and are associated with, the cardiac calcium channel have not been identified. Accordingly, we have initiated studies to purify the calcium channel from cardiac tissue using radiolabelled calcium channel antagonists as biochemical probes (2). A complex containing functional binding sites for all three types of calcium channel antagonists [1,4-dihydropyridines (DHP), phenylalkylamines, and benzothiazepines] was solubilized from chick heart membranes with digitonin[1], in a manner similar to that previously described for rat brain membranes (3), and in a form that retained characteristics of the membrane-bound sites[1]. We report here on our initial efforts to purify this complex using [^{3}H]PN 200-110 (4).

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Abbreviations: DHP, 1,4-dihydropyridines; SDS, sodium dodecyl sulfate.

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MATERIALS AND METHODS. Newborn chicks were obtained from Corn Belt Hatcheries (Forrest, IL). (+)[^{3}H]PN 200-110 was from Amersham. Nifedipine was a gift from Miles Laboratories. Digitonin was from Gallard Schlesinger (lot #3773093-5782). DEAE-Sephadex A-25 was from Pharmacia. Wheat germ agglutinin (WGA)-Sepharose 6B, molecular weight standards, and protease inhibitors were from Sigma. Hexylamine-agarose was from P-L Biochemicals.

MEMBRANE ISOLATION. Membranes were prepared as in (5) except a three-step gradient of 15%, 32%, and 40% sucrose was used and all solutions contained a protease inhibitor cocktail of 0.1 mM benzamidine, 0.02 mg/ml leupeptin, 0.007 mg/ml pepstatin, 0.01 mg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA and 0.1 mM EGTA. The gradients were centrifuged in an SW40 rotor at 38,000 rpm for 2 hrs. Membranes were harvested at the 15/32% and 32/40% interfaces as in (5). 32/40 membranes contained 2-3.5 pmols/mg protein of [^{3}H]PN 200-110 binding sites (Fig. 1). In a 6 hr isolation we obtained 20 mg 32/40 membranes/10 g tissue or 4-6 pmols DHP binding sites/g tissue. In contrast canine hearts yield 0.06-0.3 pmols of DHP binding sites/g in an 8-12 hr isolation (6-8). Thus chick hearts appear to be a more suitable source of cardiac calcium channels.

ASSAY AND SOLUBILIZATION OF [^{3}H]PN 200-110 BINDING SITES. Membrane-bound receptors were labelled with [^{3}H]PN 200-110 at 25°C for 60 min in a reaction containing 50 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF and 0.01-0.03 mg protein in a final volume of 0.5 ml. Non-specific binding was defined with 1 μM nifedipine. Membrane-bound [^{3}H]PN 200-110 was determined by rapid filtration. For solubilization studies, membranes (0.5 mg/ml) were prelabelled with 2 nM [^{3}H]PN 200-110 and sedimented at 100,000 x g for 30 min. The pellets were resuspended, solubilized with 0.5% digitonin at 0.5 mg protein/ml for 40 min at 4 ° C, and centrifuged at 130,000 x g for 30 min. The supernatants were used as the source of solubilized receptors. Protein-bound [^{3}H]PN 200-110 in the solubilized fractions was determined using polyethylene glycol precipitation (3).

PURIFICATION OF SITES PRELABELLED WITH [^{3}H]PN200-110. All procedures were performed at 4° C and all buffers contained 0.2 mM PMSF, 1 mM iodoacetamide, 0.1 mM benzamidine, 0.2 μM pepstatin A and 0.2 μM leupeptin. The digitonin extract (30-50 ml) was mixed with DEAE-Sephadex A-25 (5 ml) equilibrated with 10 mM Tris-HCl, pH 7.4, 0.1 % digitonin, 1 mM CaCl and protease inhibitors (Buffer A). The mixture was poured into a column, washed with buffer A, and eluted with a linear gradient (50 ml) of 0-0.5 M NaCl in buffer A. Active fractions were pooled and diluted 2-fold, and then passed through a hexylamine agarose column (2 ml) equilibrated with buffer B (buffer A but with Hepes instead of Tris). The column was washed with 50 mM NaCl in buffer B and eluted with a gradient (50 ml) of 0.05-0.5 M NaCl in buffer B. The peak fractions

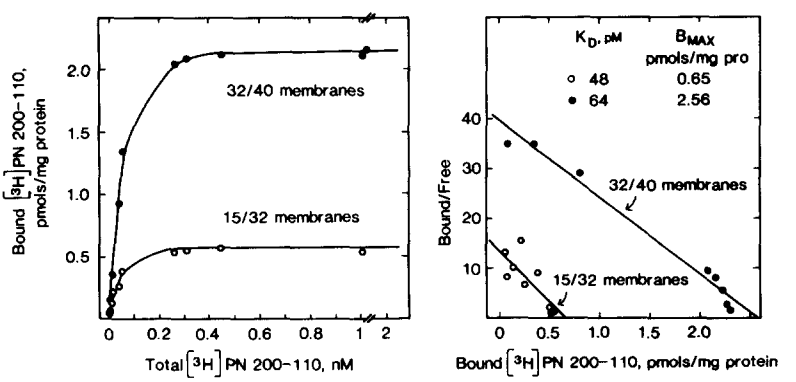

Figure 1. Saturation isotherms and scatchard analyses of [^{3}H]PN 200-110 binding to cardiac membranes. Binding was performed as described in the text. 15/32 membranes are enriched in sarcolemma markers (10), whereas 32/40 membranes are not.

were pooled and equilibrated batchwise with WGA-Sepharose 6B (1 ml) for 30 min. The gel was packed into a column, washed with 0.5 M NaCl in buffer B, and eluted with 0.2 M N-acetylglucosamine in buffer B. The pooled fractions were applied to a 38 ml linear gradient of 5-20% sucrose made in buffer A, and centrifuged in a VTi50 rotor for 105 min at 50,000 rpm. The entire purification procedure was completed in 12-15 hr. For sodium dodecyl sulfate (SDS) gel electrophoresis the samples were concentrated by ultrafiltration or by lyophilization, dissolved in SDS-diluent buffer and analyzed on 5-15% gradient gels (9,10). After fixing, the gels were stained with silver (11). Protein assays were done according to (12).

RESULTS

CHARACTERIZATION OF [^{3}H]PN 200-110 BINDING TO CHICK HEART MEMBRANES. Saturation isotherms of [^{3}H]PN 200-110 binding to chick heart membranes showed the B_{max} for the sarcolemma-enriched membranes (15/32, ref. 10) and the 32/40 membranes were 0.65 and 2.56 pmols/mg protein, respectively. The K_D values for [^{3}H]PN 200-110 were less than 0.1 nM in both membrane fractions. The reasons for the anamolous fractionation of the DHP binding sites with the non-sarcolemmal membranes are discussed elsewhere (6,8 and footnote 1). All further studies were performed using the 32/40 membranes. The membrane-bound sites labelled by [^{3}H]PN200-110 were solubilized with 0.5% digitonin (3). The characteristics of this solubilization were extremely similar to those previously observed for solubilization of sites prelabelled with [^{3}H]nitrendipine (3 and footnote 1).

PURIFICATION OF PRELABELLED [^{3}H]PN 200-110 RECEPTORS. The purification protocol that gave the best results is illustrated in Fig. 2. The activities shown in the column profiles (Fig. 2) were not corrected for dissociation of ligand. The corrected specific activities (Table 1) were calculated according to (13) except that a correction for yield was also included by calculating the recovery of the total radioactivity throughout the purification procedures. The actual yield of receptors may have been different, but cannot be directly determined because we measured prelabelled receptors. A purification of approximately 600-fold was achieved. Similar results were obtained with a protocol of WGA-Sepharose, hydroxylapatite, a second WGA-Sepharose column and sucrose density centrifugation. The specific activities (pmol/mg protein) of the fractions obtained from these steps were 33, 51, 330 and 1800, respectively. However large losses occurred when the WGA column was used as the first step.

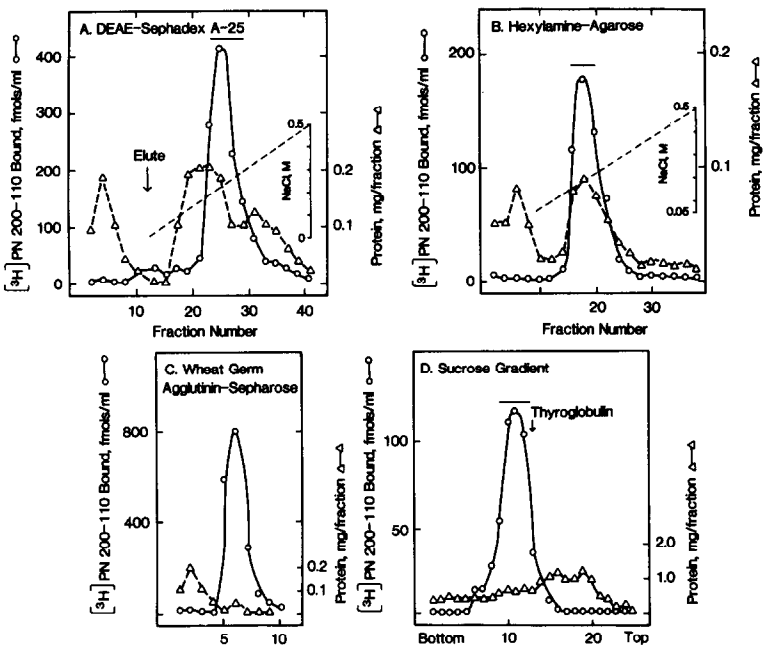

Figure 2. Column and gradient profiles of steps used for the purification of the [^{3}H]PN 200-110 receptors. The buffers and other conditions of each step are described in Materials and Methods. A. DEAE-Sephadex A-25 column. Fractions 1-12 were 5 ml; all other fractions were 1.6 ml. B. Hexylamine agarose column. Fractions 1-10 were 5 ml; the remaining fractions were 1.6 ml. C. Wheat germ agglutinin-Sepharose 6B column. Fractions 1-4 were 5 ml; fractions eluted with N-acetylglucosamine (5-10) were 0.5 ml. D. Sucrose gradient. All fractions were 1.6 ml.

Table 1

Purification of [^{3}H]PN 200-110 Receptors from Chick Heart

Step	Yield %$^+$ (of cpm)	Measured pmols receptor	% Receptors^{++} occupied	Corrected pmols receptors*	Protein, mg	Specific activity (pmols/mg protein)
Digitonin extract	100**	4.14	32	12.9	6.36	2.03
DEAE Sephadex A-25	94	1.93	15	12.3	1.40	8.76
Hexyl-amine Agarose	70	1.13	9.0	9.2	0.45	20.4
WGA-Sepharose-6B	48	0.57	4.4	6.2	0.10	62.0
Sucrose gradient	40	0.46	3.5	5.2	0.003	1595

+ "Yield" refers to the total cpms recovered. This was calculated by: (total cpms recovered in each column/total cpms applied to that column) X previous yield.

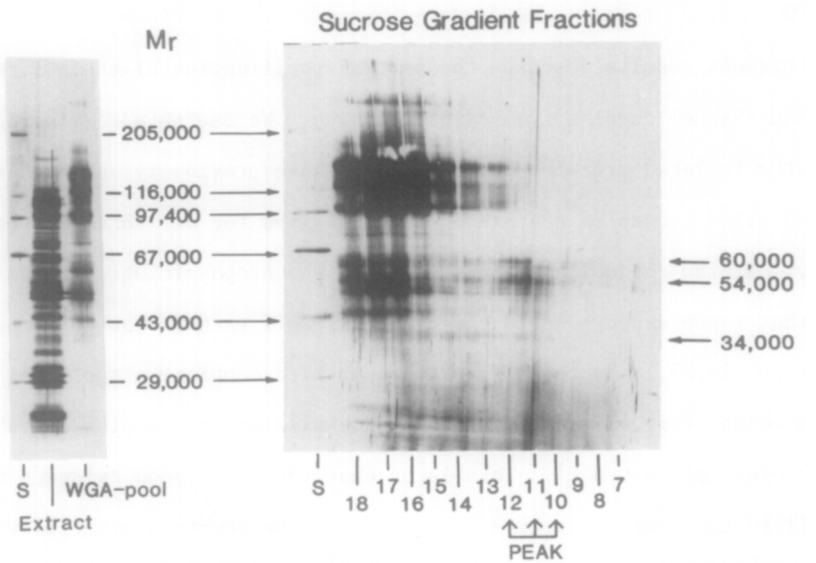

Figure 3. Silver-stained sodium dodecyl sulfate gels of fractions obtained during the purification of the [^{3}H]PN 200-110 binding sites. Fractions on the left were from the sources indicated. Fractions on the right are from the final sucrose gradient; the numbers refer to the fraction number from the gradient shown in Fig. 2D. "Peak" refers to the fractions (10-12) from the gradient containing the peak of the bound [^{3}H]PN 200-110. "S", molecular weight standards.

Analysis of the purified fractions with SDS-gel electrophoresis showed two major peptides of 60,000 and 54,000 daltons, and another peptide of 34,000 daltons (Fig. 3). These peptides were appropriately enriched in the peak fractions obtained from the sucrose gradient (Fig. 3). The 34K peptide was obscured by another peptide of slightly lower molecular weight that began to appear in the lighter fractions. The same peptide profiles were obtained in five separate purifications either using the method shown or the alternate method described above. When protease inhibitors were not included throughout the isolation procedures the 34K peptide was less apparent or absent.

++ "% receptors occupied" was calculated according to ref. 13 by: (bound cpms/original bound cpms) X 100.

* "Actual pmols receptors" was calculated by (bound cpm/% receptors occupied) X yield.

** 100% refers to the amount of radioactivity calculated to have been originally associated with a receptor. This value was obtained by determining the total and bound cpm in the membrane pellet used to prepare the digitonin extract, and was found to be approximately 50% of the total radioactivity in the digitonin extract.

DISCUSSION

The present results describe the partial purification of the DHP receptor/calcium channel complex from cardiac tissue. We use this terminology to describe the isolated proteins because: 1) we have previously shown that we have solubilized a complex containing binding sites for all three of the major classes of calcium channel antagonists[1] ; 2) electrophysiological studies suggest that these drugs bind to the channel itself rather than to a regulatory protein (14,15,); 3) the size of the purified complex on the final sucrose gradient (Fig. 2) is exactly the same size as the complex contained in the crude digitonin extract[1]. This later observation is good evidence that we have purified the complex originally present in the starting material rather than only a part of it. Further studies characterizing the purified fractions are necessary to prove this assertion.

The approach of purifying prelabelled DHP receptors is not optimal because radioligand dissociates from the receptors during the isolation. We took this approach because we found it difficult to label cardiac DHP receptors with radioligand if the sites were solubilized empty, as was done with skeletal muscle receptors (16,17). To determine the specific activities of purified prelabelled fractions it is necessary to correct for the dissociation of the ligand (13). There are obvious uncertainties with these calculations so the actual specific activities may be less (18) or greater than those shown in Table 1. However the products and specific activities obtained by purifying skeletal muscle DHP receptors with a prelabelling (13) and postlabelling technique (18) were very similar. These results showed that the prelabelling approach is valid and useful.

The structures of the calcium channels in cardiac and skeletal muscle may be both similar and different. The major peptides present in the preparations isolated from heart were 60,000, 54,000 and 34,000 daltons. The major peptides present in preparations purified from skeletal muscle were 130,000-150,000 (13,18), 53,000 (13) and 32-33,000 daltons (13,18). We did not observe a 130-150K peptide in our preparations. This result was obtained using

the purification scheme shown (Fig. 2) and also using the alternative scheme described above. The latter scheme was very similar to that used to purify sites prelabelled with [^{3}H]nitrendipine from skeletal muscle (13). A high molecular weight peptide did appear to be a contaminant in our fractions (Fig. 3). Another report, in which a photoaffinity probe was used to identify DHP binding sites, showed that a 150K peptide was not detected in cardiac membranes (19), while such a peptide was detected in skeletal muscle membranes. In contrast, in another study a 33,000 dalton protein in cardiac membranes, similar in size to a peptide observed in Fig. 3, was photolabelled with [^{3}H]nitrendipine (20). It is possible that a 150K peptide is associated with cardiac calcium channels, but in much lower amounts than in skeletal muscle. Future studies will identify drug binding sites and other characteristics of the purified DHP receptor/calcium channel complex from heart.

ACKNOWLEDGEMENT

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