0026-895X/90/020173-09$02.00/0

MOLECULAR PHARMACOLOGY, 37:173-181

Identification, Characterization, and Photoaffinity Labeling of the Dihydropyridine Receptor Associated with the L-Type Calcium Channel from Bovine Adrenal Medulla

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Received July 20, 1989; Accepted October 9, 1989

SUMMARY

The dihydropyridine receptor associated with the L-type Ca^{2+} channel in adrenal medulla membranes has been identified and characterized. [^{3}H]PN200-110 binds in a stereoselective, saturable manner to a single class of high affinity sites in adrenal medulla membranes, with a K_d of 0.1 nM and a B_{max} of 141 fmol/mg of protein. Dihydropyridines inhibited [^{3}H]PN200-110 binding with the rank order (+)-PN200-110 > nifedipine > nimodipine > nisoldipine ≥ nitrendipine > BayK8644 > (−)-PN200-110. [^{3}H]PN200-110 binding was sensitive to divalent cations, as examined by the effects of Ca^{2+}, Mg^{2+}, and the chelators ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid and EDTA. [^{3}H]PN200-110 binding was modulated by various classes of L-type Ca^{2+} channel effectors. Benzothiazepines modulated binding of [^{3}H]PN200-110 in a negative or positive manner that was temperature dependent, whereas phenylalkylamines weakly inhibited [^{3}H]PN200-110 binding. Bepridil stimulated [^{3}H]PN200-110 binding, whereas phencyclidine was without effect. The photoaffinity probe [^{3}H]azidopine labeled a single polypeptide that migrated with an apparent molecular weight of 185,000–190,000 in sodium dodecyl sulfate gel electrophoresis. The dihydropyridine receptor was found to bind specifically to wheat germ agglutinin columns. These results demonstrate the presence of a Ca^{2+} channel blocker complex in adrenal medulla. The drug receptor sites reside on a glycoprotein complex in which a polypeptide analogous to the α_1-subunit of the L-type Ca^{2+} channel from skeletal muscle has been identified.

Transmembranal flow of Ca^{2+} into the cytoplasm of excitable cells is known to regulate a wide variety of cellular processes, including muscle contraction, neurotransmitter release, and hormone secretion (1, 2). The voltage-regulated calcium channel is a major regulator of calcium influx into these cell types. The organic calcium channel blockers have been shown to bind with high affinity to sites on the L-type voltage-sensitive calcium channels and to inhibit their function in various cell types (3).

The calcium channel blockers (calcium antagonists) are divided into three main structurally dissimilar groups, (i) the DHPs, (ii) the phenylalkylamines, and (iii) the benzothiazepines (4). The availability of tritiated analogues of these drugs has allowed the identification and characterization of the receptors for these drugs on the Ca^{2+} channel structure. It is generally accepted that drugs from the three main classes of calcium antagonists bind to three distinct allosterically linked binding sites (5–7). Recent evidence indicates that the Ca^{2+} antagonist bepridil binds to a fourth site on the Ca^{2+} channel (8, 9). Recent work has also indicated that neuroleptics of the diphenylbutylpiperidine class bind to a distinct, allosterically linked, fifth site in muscle and brain tissue (10–12). Hydrophobic cations of the amiloride class have also been suggested to bind to a sixth site on the voltage-sensitive Ca^{2+} channel (13). Together these distinct, allosterically coupled, drug binding sites identify the Ca^{2+} entry blocker complex associated with the L-type Ca^{2+} channel in muscle and brain (12).

Ca^{2+} antagonists and agonists have been shown to modulate catecholamine secretion from perfused adrenal glands, cultured chromaffin cells, and pheochromocytoma (PC12) cell lines (14–20). It is believed that these drugs mediate their effects by acting on the voltage-sensitive Ca^{2+} channel. However, the pharmacological as well as biochemical properties of the types of Ca^{2+} channels involved in catecholamine secretion in the adrenal gland remain to be determined. In view of the availability of the various high affinity radioligands for the L-type

B.S.T. is a scholar of the Ontario Heart and Stroke Foundation B.J.M. is a recipient of a predoctoral traineeship from the Ontario Heart and Stroke Foundation. This work was supported by grants from the Ontario Heart and Stroke Foundation and Medical Research Council of Canada.

ABBREVIATIONS: DHP, 1,4-dihydropyridine; SDS-PAGE, sodium dodecyl sulfate; WGA, wheat germ agglutinin; T-tubule, transverse tubule; EGTA, ethylene glycol bis-(β-aminoethyl ether-*N,N,N′,N′*-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PCP, phencyclidine; K_d, apparent dissociation constant; B_{max}, maximal binding capacity; K_i, inhibition constant; IC_{50}, concentration causing half-maximal inhibition; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis.

Ca^{2+} channel, it should be possible to identify and characterize the Ca^{2+} channel complex from the adrenal gland by radioligand binding studies and photoaffinity labeling.

In this study we have employed the DHP [^{3}H]PN200-110 to directly identify and characterize the DHP receptor in membranes prepared from the medulla of the bovine adrenal gland. We also establish the existence of phenylalkylamine, benzothiazepine, and bepridil receptors that are allosterically linked to the DHP site in a manner similar to that described for other tissues (6, 7, 21). We identified the DHP-binding polypeptide of the voltage-gated Ca^{2+} channel by photoaffinity labeling with [^{3}H]azidopine. Taken together, these results demonstrate the existence of a Ca^{2+} entry blocker complex in the bovine adrenal gland that is similar to that described in muscle and brain. Our results also show that the DHP binding polypeptide in the bovine adrenal medulla is a M_r 185,000–190,000 polypeptide analogous to the α_1 subunit of the L-type Ca^{2+} channels from muscle and brain.

Experimental Procedures

Materials. [^{3}H]PN200-110 (70 Ci/mmol) and [^{3}H]azidopine (40 Ci/mmol) were purchased from Amersham (Oakville, Ontario, Canada). Unlabeled (±)-PN200-110, (+)-PN200-110, and (−)-PN200-110 were gifts from Sandoz Laboratories, Inc. Nifedipine, nitrendipine, nimodipine, and BayK8644 were kind gifts from Drs. D. Frankel and Dr. A. Scrabine of Miles Laboratories, Inc. Diltiazem was supplied by Dr. N. Mhaer of Nordic Laboratories, Inc. Verapamil and gallopamil were provided by Knoll Pharmaceutical Canada, Inc. Unlabeled desmethoxyverapamil was purchased from Amersham. Fresh bovine adrenal glands were purchased from Abattoir Savage (St. Albert, Ontario, Canada). WGA-Sepharose, *N*-acetyl glucosamine, and SDS-PAGE molecular weight markers were purchased from Sigma. Protease inhibitors were purchased from Sigma and Boehringer Mannheim. All other chemicals were of analytical grade or the highest grade available.

Membrane preparation. Bovine adrenal glands were obtained from a local slaughterhouse (St. Albert, Ontario, Canada) within 5 min after death, placed on ice, and immediately transported to the laboratory. Membranes were prepared by the method of Rogers *et al.* (22). All steps were carried out at 0–4°, and all buffers contained the following protease inhibitors: 0.1 mM PMSF, 1 μM pepstatin A, 1 mM iodoacetamide, 0.75 mM benzamidine, 1 μg/ml trypsin inhibitor, and 1 μg/ml leupeptin. Briefly, the medulla was dissected free from cortical tissue, minced with a Waring blender (2 × 20 sec) and homogenized with a glass-Teflon homogenizer in 10 volumes of ice-cold 50 mM Tris·HCl, pH 7.4 (Buffer A). Particulate matter was removed by centrifugation at 1000 × *g* for 20 min. The pellets were discarded and the supernatant was centrifuged at 26,000 × *g* for 30 min. The resulting membrane pellet was washed as above and centrifuged at 26,000 × *g* for 30 min. The final pellets were resuspended in Buffer A at a concentration of 2 mg/ml, quick frozen in liquid nitrogen, and stored at −90°.

[^{3}H]PN200-110 binding assays. In saturation binding experiments, 0.2 mg of adrenal medullary membranes were incubated in triplicate with increasing concentrations of [^{3}H]PN200-110 (0.02–2.0 nM) in a final volume of 1 ml in Buffer A. Binding reactions were carried out at equilibrium (90 min) and were terminated by rapid vacuum filtration using a 12-well Skatron cell harvester (Skatron, Inc., Sterling VA) over glass fiber "receptor grade" filtermats (Skatron). Filters were washed with 9 ml of Buffer A for 15 sec (wash fluid passing over filters in 1.5 sec and placed in scintillation mini-vials (skatron) with 3.0 ml of Ecolume scintillation cocktail (ICN, Irvine CA). Vials were monitored for tritium, after 20-hr equilibration, in a Beckman LS7800 liquid scintillation counter at approximately 40% efficiency.

For competition experiments, reactions were carried out in duplicate, as described above, with a fixed concentration of [^{3}H]PN200-110 along with varying concentrations of competing ligand. Reactions were terminated by rapid vacuum filtration as described above.

In all binding experiments, specific [^{3}H]PN200-110 binding was defined as that displaced by 1 μM unlabeled nifedipine. This value was taken from nifedipine versus [^{3}H]PN200-110 competition curves where 1 μM unlabeled nifedipine inhibited greater than 95% of [^{3}H]PN200-110 binding. Binding data were analyzed by the nonlinear least squares curve-fitting program LIGAND (23). Binding to solubilized receptor was by PEG precipitation, as described by Curtis and Catterall (24).

Photolabeling of adrenal medulla membranes with [^{3}H]azidopine. Adrenal membranes (200 mg) were diluted to 2 mg/ml in Buffer A, containing 0.1 mM PMSF and 1 μM pepstatin A, and incubated with 5 nM [^{3}H]azidopine for 90 min, in the presence or absence of 1 μM unlabeled nifedipine. The membranes were pelleted by centrifugation at 120,000 × *g* for 30 min, resuspended to 2 mg/ml in Buffer A with protease inhibitors described above, and irradiated on ice for 10 min with a handheld UVSL 56 lamp at 2 cm distance. An equal volume of 2% digitonin in Buffer A containing 1 M NaCl and the full complement of protease inhibitors employed in membrane preparation was added and the mixture was rotated at 4° for 30 min. Insoluble material was pelleted by centrifugation at 120,000 × *g* for 45 min. Soluble protein was then applied to 40 ml of WGA-Sepharose preequilibrated in 50 mM Tris·HCl, pH 7.4, 0.5 M Nacl, 0.1 mM PMSF, 1 μM pepstatin A, 1 mM iodoacetamide, 0.75 mM benzamidine, 1 μg/ml trypsin inhibitor, and 1 μg/ml leupeptin (Buffer B), containing 1% digitonin, and was rotated for 1.5 hr at 4°. Breakthrough was collected and the column was washed with first Buffer B containing 1% digitonin and then Buffer B containing 0.1% digitonin. The column was then eluted by incubation with Buffer B containing 0.1% digitonin and 0.5 M *N*-acetyl glucosamine for 2–3 hr. Aliquots of all fractions were counted directly or subjected to PEG precipitation to monitor bound [^{3}H]azidopine. Peak fractions were concentrated by ultracentrifugation (Centricon-30, Amicon Inc.) and immediately processed for SDS-PAGE. Skeletal muscle T-tubule DHP receptor was photolabeled and enriched as above except that membranes (20 mg) were incubated with 50 nM [^{3}H]azidopine, solubilized, applied to a 2-ml WGA-Sepharose column, and subsequently eluted with 200 mM N-acetyl glucosamine.

SDS-PAGE. Samples were processed for SDS-PAGE by heating to 90° for 1 min in the presence of 2% SDS with 0.1 mM PMSF, 1 μM pepstatin A, 1 mM iodoacetamide, 0.75 mM benzamidine, 1 μg/ml leupeptin, 1.0 mM EDTA, 60 mM Tris·HCl, pH 6.8, and 10% glycerol. Samples were then applied to 5–10% linear gradient polyacrylamide slab gels (10 cm) or 7.0% mini-gels (Bio-Rad) in the discontinuous buffer system described by Laemmli (25). Mini-gels were silver stained to visualize proteins, as per Morrisey (26). Gradient gels were cut into 2.0-mm strips and digested overnight at 60° in 30% H_2O_2. Scintillation fluid (Ecolume, ICN) was added and the samples were allowed to equilibrate for 72 hr before radioactivity was measured (10 min/vial) in a Beckman LS7800 liquid scintillation counter.

Protein determination. Protein concentrations were estimated either by the method of Lowry *et al.* (27) or by the method of Bradford (28), using the Bio-Rad kit and bovine serum albumin as the standard.

Results

Time and tissue concentration dependence of [^{3}H]PN200-110 binding to membrane preparations of bovine adrenal medulla. The specific binding of [^{3}H]PN200-110 was examined in membranes prepared from the bovine adrenal medulla. Fig. 1A shows the time dependence of [^{3}H]PN200-110 binding to adrenal medulla membrane preparations. The binding of [^{3}H]PN200-110 was rapid, reaching a maximum at 50 min of incubation and remaining stable for at least 2 hr at 20°. The tissue concentration and wash dependence of [^{3}H]PN200-110 binding was investigated (Fig. 2). Specific [^{3}H]PN200-110 binding increased linearly with increasing protein concentration from 0.05 to 0.4 mg/ml. The [^{3}H]PN200-

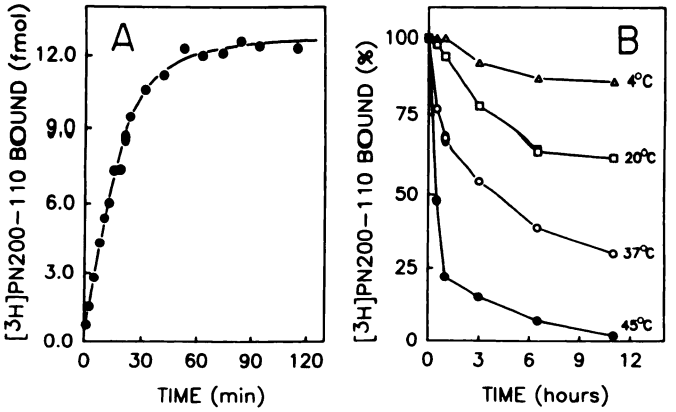

Fig. 1. Time dependence and heat stability of [^{3}H]PN200-110 binding to bovine adrenal membranes. A, Bovine adrenal membranes (0.2 mg of protein/ml) were incubated with [^{3}H]PN200-110 (0.1 nM) at 20°, and the incubation was terminated at various time intervals by rapid vacuum filtration, as described in Experimental Procedures. Specific [^{3}H]PN200-110 binding was determined as the difference between the [^{3}H]PN200-110 bound in the absence and presence of 1 μM unlabeled nifedipine. B, Membranes were incubated at the temperatures indicated and [^{3}H]PN200-110 binding was measured at various time intervals at 20° as described above. Specific binding was calculated for each time point and expressed as a percentage of control binding (measured at time 0 hr at 20°).

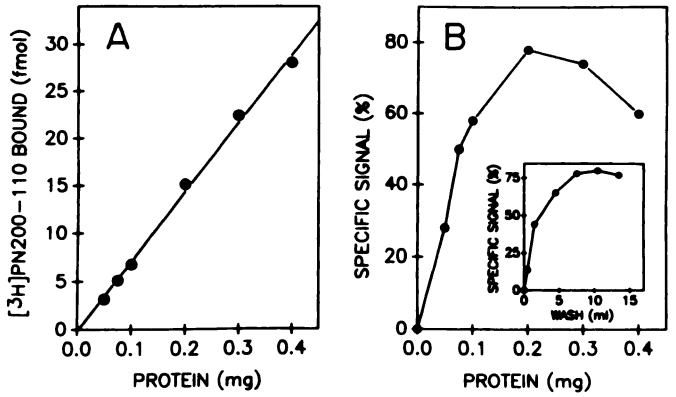

Fig. 2. Tissue and filter-wash dependence of [^{3}H]PN200-110 binding to bovine adrenal membranes. For tissue dependence (A), bovine adrenal membranes (0.05–0.4 mg of protein/ml) were incubated in the presence of 0.1 nM [^{3}H]PN200-110 for 90 min before termination, as described in Experimental Procedures. Nonspecific binding was determined at each point in the presence of 1 μM unlabeled nifedipine and was subtracted from total binding to yield specific binding. Specific signal in B is determined as specific [^{3}H]PN200-110 bound/total [^{3}H]PN200-110 bound × 100 for each point. For wash curve (B, *inset*), bovine adrenal membranes (0.2 mg of protein/ml) were incubated with 0.1 nM [^{3}H]PN200-110 for 90 min. Binding reactions were terminated and washed with increasing volumes of filter wash as indicated. *Points* for each experiment are the mean of triplicate determinations and are a representative experiment.

110 binding signal (cpm bound, specific/cpm bound, total × 100) increased with increasing protein concentration reaching a maximum of 75–80% at 0.2 mg/ml protein (Fig. 2B). The effect of volume of filter wash on the measurement of [^{3}H]PN200-110 binding is illustrated in Fig. 2B (*inset*). Maximal specific signal was attained with a wash volume of 9 ml. Increasing the volume of wash buffer to 15 ml did not increase the specific [^{3}H]PN200-110 binding signal. In all subsequent experiments, [^{3}H]PN200-110 binding was measured after 60 min of incubation with 0.2 mg/ml protein. Filters were subsequently washed with 9.0 ml of wash buffer.

Temperature-dependent inactivation of [^{3}H]PN200-110 binding. The heat lability of the [^{3}H]PN200-110 binding site is illustrated in Fig. 1B. Adrenal medulla membranes were incubated for various time intervals at the temperatures indicated, after which aliquots were removed and measured for [^{3}H]PN200-110 binding at 20°. As would be expected for a proteinaceous receptor, [^{3}H]PN200-110 binding was rapidly inactivated by preincubation of the membranes at 45° (50% inactivation by 20 min). The [^{3}H]PN200-110 binding site was stable at 4°, with only a 12% decrease in [^{3}H]PN200-110 binding after incubation at 4° for 12 hr. The [^{3}H]PN200-110 binding site showed intermediate sensitivity to preincubation at 20° and 37° (Fig. 1B).

Saturation binding of [^{3}H]PN200-110 to adrenomedullary membranes. Saturable binding of [^{3}H]PN200-110 to membranes is shown in Fig. 3. Transformation of the saturation curve in the Scatchard plot (Fig. 3, *inset*) generated a linear curve, implying that [^{3}H]PN200-110 binds to a single population of homogeneous sites. At 20°, [^{3}H]PN200-110 binds to adrenal medulla membranes with a specific activity of 141 fmol/mg of protein and a binding affinity, K_d, of 0.1 nM. In order to examine the effect of incubation temperature on [^{3}H]PN200-110 binding, saturation isotherms were also performed at 4° and 37°. Scatchard plots derived from both saturation curves are illustrated in Fig. 4. Incubation at 37° decreased specific [^{3}H]PN200-110 binding by approximately 63% and increased the binding affinity to 0.05 nM, whereas incubation at 4° had little effect on binding parameters.

Stereospecificity and pharmacological profile of [^{3}H]PN200-110 binding to membranes of the adrenal medulla. Stereospecific binding of [^{3}H]PN200-110 to adrenal medullary membranes was demonstrated in competition assays performed against stereoisomers of PN200-110 (Fig. 5). Unlabeled (+)-PN200-110 potently and completely inhibited [^{3}H]PN200-110 binding, with a K_i of 0.12 nM. Unlabeled (−)-PN200-110 was 100-fold less potent than the dextroisomer, (K_i = 30 nM). The ability of several other unlabeled DHP-type Ca^{2+} antagonists to inhibit [^{3}H]PN200-110 binding was also

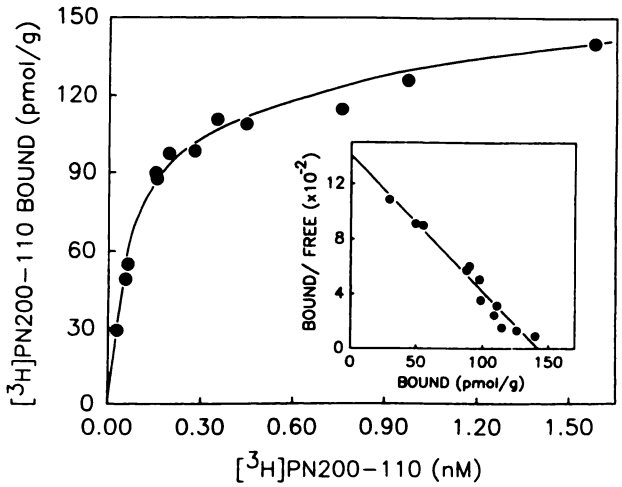

Fig. 3. Saturation of [^{3}H]PN200-110 binding to membranes of the bovine adrenal medulla. Bovine adrenal membranes (0.2 mg of protein/ml) were incubated for 90 min with increasing concentrations of [^{3}H]PN200-110 (0.02–1.5 nM) and assayed for specific binding, as described in Experimental Procedures. Nonspecific binding was defined by 1 μM unlabeled nifedipine. The data were analyzed by the nonlinear least square fitting program LIGAND. *Inset*, representation of the binding as a Scatchard plot. B_{max} and K_d values are given in the text.

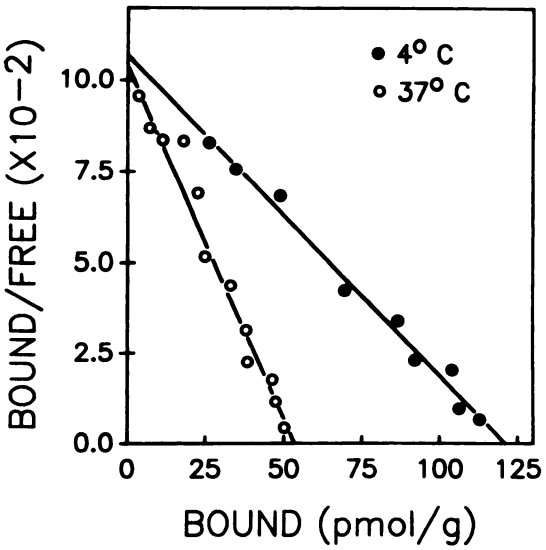

Fig. 4. Effect of temperature on the binding of [^{3}H]PN200-110 to bovine adrenal membranes. Bovine adrenal membranes (0.2 mg of protein/ml) were incubated for 90 min at 4° (●) or 37° (○) with increasing concentrations of [^{3}H]PN200-110 (0.02–1.5 nM), and assayed for specific binding as described in Experimental Procedures. Nonspecific binding was defined by 1 μM unlabeled nifedipine. The data are presented in the form of a Scatchard plot. B_{max} and K_d values are given in the text.

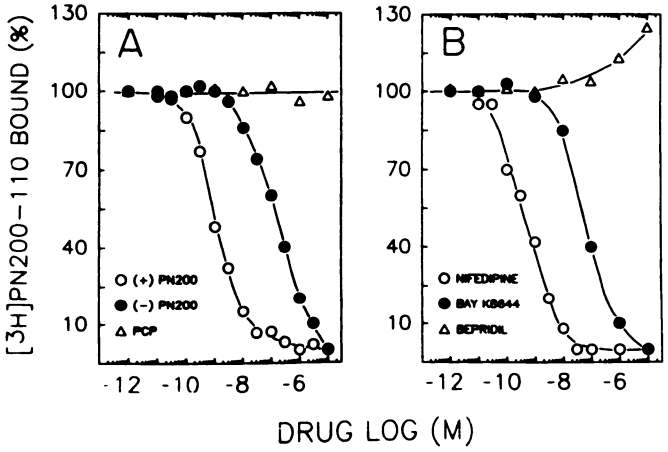

Fig. 5. Stereospecificity and pharmacological profile of [^{3}H]PN200-110 binding to bovine adrenal membranes. Bovine adrenal membranes (0.2 mg of protein/ml) were incubated with [^{3}H]PN200-110 (0.12 nM) along with increasing concentrations of unlabeled compounds for 90 min at 20°. Reactions were terminated by rapid vacuum filtration, as described in Experimental Procedures. Each *point* is the mean of triplicate determinations. Standard error was typically less than 5%. Inhibition constants for competing compounds under these experimental conditions are listed in Table 1.

examined (see Fig. 5B and Table 1). The DHPs tested were potent and complete inhibitors of [^{3}H]PN200-110 binding with the rank order (+)-PN200-110 > nifedipine > nitrendipine ≥ nisoldipine > nimodipine ≫ (−)-PN200-110. The ability of the DHP-type Ca^{2+} channel agonist BayK8644 to modulate [^{3}H]PN200-110 binding is also illustrated in Fig. 5B. As reported for other tissues, BayK8644 was much less potent at inhibiting [^{3}H]PN200-110 binding than the DHP antagonists (K_i = 20 nM). The ability of unlabeled PCP to displace [^{3}H]PN200-110 binding was also examined in Fig. 5A. PCP, a dissociative anaesthetic, had no effect on [^{3}H]PN200-110 binding over the entire concentration range examined. The calcium channel

TABLE 1

Inhibition of [^{3}H]PN200-110 binding to bovine adrenal membranes by DHP-type calcium antagonists

Bovine adrenal membranes were assayed for [^{3}H]PN200-110 binding, as described in Experimental Procedures, with varying concentrations of competing ligand. Nonspecific binding was defined by 1 μM unlabeled nifedipine.

DHP	K_i
	nM
(+)-PN200-110	0.12
Nifedipine	0.16
Nimodipine	0.18
Nitrendipine	0.19
Nisoldipine	0.19
BayK8644	20
(−)-PN200-110	30

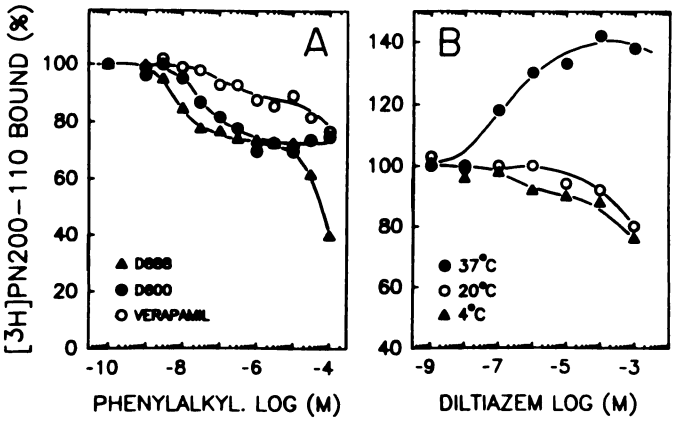

Fig. 6. Modulation of [^{3}H]PN200-110 to bovine adrenal membranes by diltiazem and phenylalkylamines. Bovine adrenal membranes (0.2 mg of protein/ml) were incubated with [^{3}H]PN200-110 (A) in the presence of increasing concentrations of unlabeled verapamil (○), D600 (●), or (−)-desmethoxyverapamil (▲) for 90 min or (B) in the presence of increasing concentrations of unlabeled diltiazem at 4° (▲), 20° (○), or 37° (●). Specific binding was calculated and expressed as a percent of control binding in the absence of diltiazem at each temperature, (B) or in the absence of phenylalkylamines (A). *Points* for each experiment are the mean of triplicate determinations and are a representative experiment.

blocker bepridil stimulated [^{3}H]PN200-110 binding by 25% at 10 μM, half-maximal stimulation occurring at approximately 1 μM (Fig. 5B).

Allosteric modulation of [^{3}H]PN200-110 binding. Diltiazem has been reported to regulate [^{3}H]PN200-110 binding to the L-type calcium channel in various tissues in a temperature-dependent manner. In order to examine the ability of diltiazem to modulate [^{3}H]PN200-110 binding to adrenal medullary membranes, competition curves were performed against unlabeled diltiazem at 4°, 20°, and 37° (Fig. 6B). Diltiazem had little effect on [^{3}H]PN200-110 binding at 4° and 20°. At high concentrations (1 mM), diltiazem inhibits [^{3}H]PN200-110 binding by 20–25% at these temperatures. At 37°, diltiazem stimulated [^{3}H]PN200-110 binding in a dose-dependent manner. Maximal stimulation of [^{3}H]PN200-110 binding occurred in the presence of 0.1 mM diltiazem. Scatchard analysis of saturation curves performed at 37° in the presence and absence of diltiazem showed that diltiazem increased the number of binding sites (B_{max}) detected, with no significant change in the affinity of [^{3}H]PN200-110 for its receptor (data not shown).

Competition data for phenylalkylamine-type Ca^{2+} channel blockers demonstrate that the phenylalkylamines only partially inhibit [^{3}H]PN200-110 binding (Fig. 6A). Desmethoxyverapa-

mil was the most potent, inhibiting the binding of [^{3}H]PN200-110 in a biphasic manner. Desmethoxyverapamil inhibited 25% of [^{3}H]PN200-110 binding with an IC_{50} of 10 nM; higher concentrations (0.1 mM) of desmethoxyverapamil inhibited a further 35% of the total [^{3}H]PN200-110 bound. D600 inhibited only 25% of the bound radiolabel, with an IC_{50} of 30 nM. Verapamil was the least potent of the phenylalkylamines tested, inhibiting only 10% of the binding at 10 μM; the maximal inhibition was observed at 1 mM.

Modulation of [^{3}H]PN200-110 binding by divalent ions. The ability of exogenously added divalent cations to modulate [^{3}H]PN200-110 binding to adrenal medulla membranes is illustrated in Fig. 7A. The addition of Ca^{2+} ions stimulated [^{3}H]PN200-110 binding by approximately 30% at 1 mM, with half-maximal stimulation occurring at 0.3 mM. The addition of millimolar concentrations of Mg^{2+} ions inhibited [^{3}H]PN200-110 binding 20% at 10 mM.

In a separate series of experiments, the effects of divalent ion-chelating agents on [^{3}H]PN200-110 binding were examined (Fig. 7B). The addition of increasing concentrations of EDTA or EGTA to the binding reactions resulted in a biphasic response. Inhibition of [^{3}H]PN200-110 binding at low concentrations of the chelators was followed by a return to control levels at higher concentrations.

Effect of sulfhydryl-modifying agents on [^{3}H]PN200-110 binding. The possible role of disulfide bonds and free sulfhydryl groups in [^{3}H]PN200-110 binding was investigated. Adrenal medulla membranes were preincubated in the presence of increasing concentrations of modifying reagents for 20 min, after which specific [^{3}H]PN200-110 binding was measured at 20°, as described in Experimental Procedures. The alkylating agents *N*-ethylmaleimide and iodoacetamide inhibited [^{3}H]PN200-110 binding by 85% and 40%, respectively (Fig. 8). Preincubation of adrenal medulla membranes in the presence of increasing concentrations of the reducing agent DTT resulted in a dose-dependent decrease in [^{3}H]PN200-110 binding (Fig. 8). DTT inhibited [^{3}H]PN200-110 binding to approximately 85%, with an IC_{50} = 1 mM.

Photoaffinity labeling and glycoprotein nature of the bovine adrenal DHP receptor. In order to identify the DHP-binding polypeptide present in adrenal medulla membranes, we have employed [^{3}H]azidopine, an aryl-azido derivative of the DHP-type calcium blockers that binds covalently to the receptor in the presence of UV light (29). [^{3}H]azidopine has proven to be a useful photoaffinity probe for identifying the DHP-binding subunit of the L-type Ca^{2+} channel in muscle and brain (5, 30–37). The low abundance of DHP receptors in adrenal medulla membranes precluded the identification of the DHP-binding component directly in membranes by photoaffinity labeling. In order to circumvent this problem, membrane preparations were covalently labeled with [^{3}H]azidopine and a procedure was established for the enrichment of the labeled polypeptides. Due to the glycoprotein nature of the DHP receptor from muscle and brain (24, 38), we examined whether the DHP binding sites from adrenal medulla membranes could be enriched by WGA-Sepharose lectin affinity chromatography. Labeled membranes solubilized in 1% digitonin were applied to a WGA-Sepharose column. The column was extensively washed with buffer to remove nonspecifically bound protein, and bound

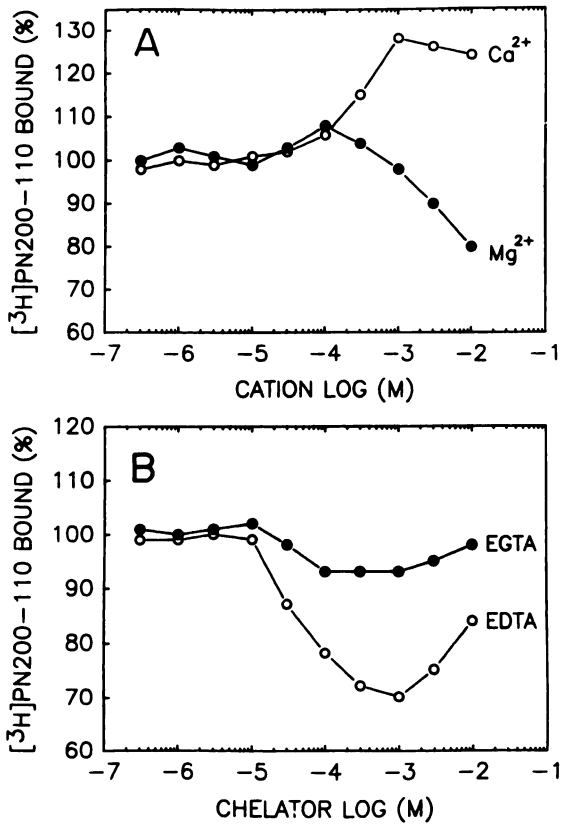

Fig. 7. Effect of divalent ions on the binding of [^{3}H]PN200-110 to membranes of the bovine adrenal medulla. Bovine adrenal membranes (0.2 mg of protein/ml) were incubated with 0.15 nM [^{3}H]PN200-110 at 20° until equilibrium was achieved in the presence of increasing concentrations of Ca^{2+} (○) or Mg^{2+} (●) (A) or EGTA (●) or EDTA (○) (B). Reactions were terminated as described in Experimental Procedures. Nonspecific binding was defined by 1 μM unlabeled nifedipine. *Points* are the mean of triplicate determinations.

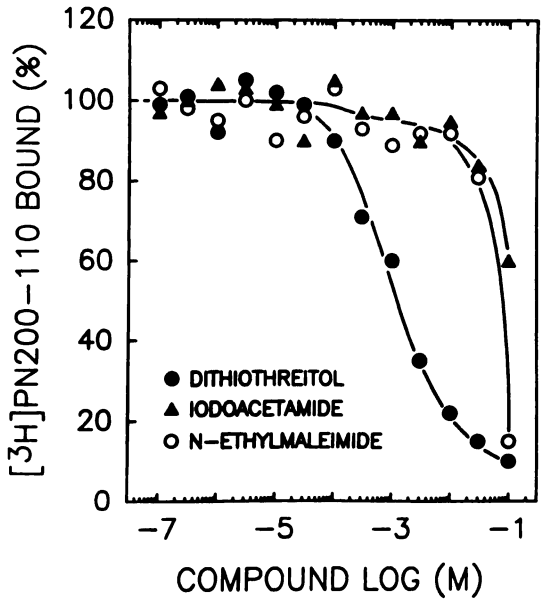

Fig. 8. Effect of sulfydryl group-modifying reagents on the binding of [^{3}H]PN200-110 to bovine adrenal membranes. Bovine adrenal membranes were preincubated at 20° in the presence of increasing concentrations of modifying reagents, for 30 min. Membranes were then assayed for [^{3}H]PN200-110 binding at 20° in the presence of 0.12 nM of radiolabeled ligand. Nonspecific binding was defined by 1 μM unlabeled nifedipine. One hundred percent binding corresponds to specific [^{3}H]PN200-110 binding measured after a 30-min preincubation in the absence of modifying reagents.

receptor was eluted with *N*-acetyl glucosamine. Fig. 9A shows that the [^{3}H]azidopine-labeled receptors, as determined by PEG precipitation, were specifically retained by the WGA column, suggesting that these DHP binding sites are associated with a glycoprotein complex. Eluted receptor was concentrated by ultrafiltration and subjected to analysis by SDS-PAGE (Fig. 9B). The gels were silver stained to visualize proteins (Fig. 9B) or subjected to gel slice analysis to monitor the incorporation of [^{3}H]azidopine. Fig. 10 (*top*) shows that [^{3}H]azidopine was incorporated into a polypeptide of apparent molecular weight 185,000–190,000 in the adrenal medulla. Labeling of this polypeptide was inhibited by the inclusion of 1 μM unlabeled nifedipine during UV irradiation. Under similar conditions, [^{3}H]azidopine is specifically incorporated into a polypeptide of M_r 170,000 in skeletal muscle T-tubules (Fig. 10, *bottom*). The apparent molecular weight of the [^{3}H]azidopine-labeled polypeptide from adrenal medulla was larger than the DHP-binding polypeptide labeled in skeletal muscle membranes. The mobility of the DHP-binding polypeptide in the adrenal medulla was not altered by electrophoresis under reducing conditions (10 mM DTT), although the recovery of [^{3}H]azidopine was decreased (data not shown).

Discussion

In this study we have identified and characterized in detail the DHP binding site in bovine adrenal membranes. We used radioligand binding and photoaffinity labeling to identify the L-type Ca^{2+} channel-DHP receptor complex. [^{3}H]PN200-110 bound to a single class of DHP binding sites in adrenal medulla membrane preparations. The DHP receptor coexists in a complex with the benzothiazepine and phenylalkylamine binding sites. All three receptor types are coupled in a fashion that displays both positive and negative allosteric interactions. These characteristics of drug binding are analogous to those reported for the L-type Ca^{2+} channel from muscle and brain. Inasmuch as voltage-sensitive Ca^{2+} channels are known to exist in the adrenal medulla, the presence of these receptors is consistent with the hypothesis that these sites exist on the voltage-gated Ca^{2+} channel complex.

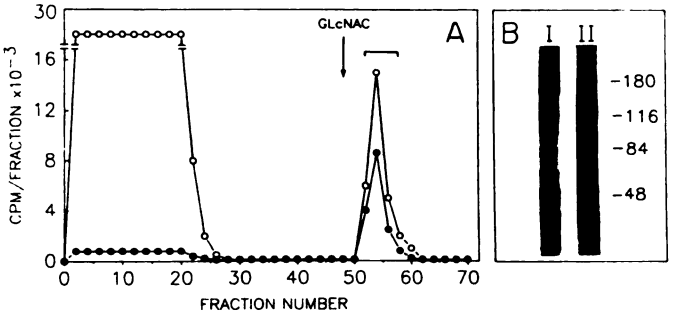

Fig. 9. Elution profile of [^{3}H]azidopine-labeled DHP receptor from WGA-Sepharose. A, Bovine adrenal membranes were covalently labeled with [^{3}H]azidopine, solubilized in digitonin, and applied to WGA-Sepharose, as described in Experimental Procedures. Breakthrough fractions, 1–20 (10 ml/fraction). The column was washed with Buffer B containing 0.3% digitonin and 0.5 M NaCl, yielding fractions 21–48 (10 ml/fraction). The column was then eluted (fractions 49–70) with the above buffer supplemented with 0.5 M *N*-acetylglucosamine (3 ml/fraction). Aliquots (0.1 ml) were either counted directly (○) to measure total [^{3}H]azidopine or subjected to PEG precipitation (●) to monitor protein-bound [^{3}H]azidopine. *Bar* indicates fractions pooled. B, Silver stained SDS-PAGE of 2 μg of adrenal membranes (*I*), or 2 μg of peak elute fraction (*II*).

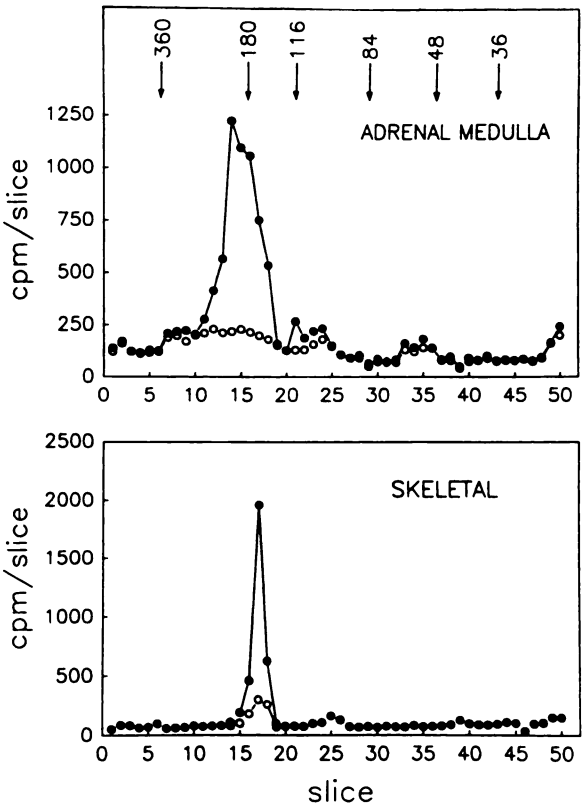

Fig. 10. Photoaffinity labeling of DHP receptor in bovine adrenal membranes and skeletal muscle T-tubules with [^{3}H]azidopine. Bovine adrenal membranes (*top*) and skeletal muscle membranes (*bottom*) were covalently labeled with [^{3}H]azidopine, solubilized in digitonin, and enriched by WGA-Sepharose chromatography, as described in Experimental Procedures. Labeled receptor was resolved by 5–15% SDS-PAGE under nonreducing conditions (10 mM *N*-ethylmaleimide) and the gel was sliced and analyzed for radioactivity as described in Experimental Procedures. Total [^{3}H]azidopine binding (●) and [^{3}H]azidopine binding in the presence of 1μM nifedipine. *Arrows* indicate the location of molecular weight markers. Numbers represent $M_r \times 10^{-3}$.

The results indicate that the binding of DHPs to the adrenal medulla membrane is to a proteinaceous component and is a specific interaction. Stereoisomers of PN200-110 inhibit binding with the rank of potency expected from their ability to block L-type calcium channels, (+)-PN200-110 much more potently than (−)-PN200-110. PN200-110 has also been shown to inhibit the K^+-induced catecholamine release from perfused cat adrenal glands with the same stereospecificity (20). The IC_{50} for (+)-PN200-110 was subnanomolar, in agreement with its K_d reported here (20). Furthermore, all other Ca^{2+} entry blockers, as well as the Ca^{2+} channel agonist BayK8644, affect [^{3}H]PN200-110 binding in a manner that is characteristic of binding to the L-type Ca^{2+} channel structure. In this regard, BayK8644 has also been shown to facilitate catecholamine release from PC12 cells as well as perfused cat adrenal glands (18, 19). Phenylalkylamines inhibit DHP binding in a negative allosteric manner, whereas the benzothiazepine diltiazem regulated DHP binding in a positive allosteric manner. Raising the temperature from 20° to 37° radically alters the coupling between individual receptor binding domains and changes the binding properties of the DHP site. Similar results have been reported for the DHP binding site associated with the L-type calcium channel from different tissues (39–41).

Although the overall characteristics of the Ca^{2+} channel-

receptor complex from the adrenal medulla appear to be similar to that present in brain and muscle membranes, our results also indicate some subtle differences in these receptors. For example, the agonist BayK8644 inhibited DHP binding in the adrenal medulla 5-fold less potently than in cardiac muscle (42, 43). Ca^{2+} ions stimulated and Mg^{2+} ions inhibited [^{3}H]PN200-110 binding in adrenal medulla, whereas in cardiac muscle Mg^{2+} ions dramatically stimulated DHP binding and Ca^{2+} had a marginal stimulatory effect (44).

The presence of divalent cations has been shown to be a requirement for high affinity binding of DHPs to brain and muscle (45–50). The presence of EGTA in the binding reactions reduced [^{3}H]PN200-110 binding by 10%, whereas the presence of EDTA was capable of reducing binding by 30%. This differential sensitivity of DHP binding to EDTA and EGTA has been seen in skeletal (45, 51) and cardiac muscle (52) membranes preparations. The chelator-induced inhibition could be reversed by the addition of exogeneous Ca^{2+} ions (data not shown), indicating that they mediate their inhibition through ion chelation. Reversibility of EGTA and EDTA effects by ions has been shown in brain and skeletal muscle membranes (46, 48). It should be noted that DHP binding in adrenal medulla is much less sensitive to ion chelation than the brain or muscle DHP receptor.

The preincubation of bovine adrenal membranes with various concentrations of DTT resulted in a dose-dependent reduction in [^{3}H]PN200-110 binding (IC_{50} = 1 mM), indicating the possible role of disulfide bonds in the binding of [^{3}H]PN200-110. DTT was previously shown to completely and potently inhibit the binding of [^{3}H]nitrendipine to skeletal muscle T-tubule membranes, with a K_i of 50 μM (53). DTT has no significant effect on the binding of [^{3}H]PN200-110 to cardiac sarcolemmal membranes (54). These results indicate possible differences in the chemical nature or molecular configuration of the DHP-binding polypeptide in different tissues.

The apparent dissociation constant of approximately 0.1 nM is consistent with the subnanomolar affinity of [^{3}H]PN200-110 for its binding site on the L-type Ca^{2+} channel in muscle and brain preparations (50). The affinity of [^{3}H]PN200-110 for its binding site in adrenal medulla membranes is similar to its affinity measured in membranes prepared from PC12 cells, although a second low affinity DHP binding site was also reported (55). We did not detect a low affinity binding site in bovine adrenal medulla. It should be noted that the IC_{50} for the effect of PN200-110 on catecholamine release is more than 200 times larger than the K_d of binding (55). This disparity may be due to voltage-dependent binding of DHPs.

It is well established that phenylalkylamines bind to a site distinct from the DHP binding site and modulate DHP binding in a negative allosteric manner in a variety of tissues (7, 56). Our results indicate that the phenylalkylamines only partially inhibit the binding of [^{3}H]PN200-110 to adrenal medulla membranes, with the rank order (−)-desmethoxyverapamil > D600 > verapamil, indicating that they are modulating DHP binding in a noncompetitive manner. The inhibition of DHP binding by phenylalkylamines in adrenal medulla membranes is different from that observed in skeletal muscle membranes, where they are complete and potent inhibitors of DHP binding (50, 57).

The benzothiazepine diltiazem can modulate the binding of [^{3}H]PN200-110 to adrenal medulla membranes in a temperature-dependent manner. Diltiazem inhibited [^{3}H]PN200-110 binding by up to 20% when binding reactions were carried out at 4° or at 20°. At 37°, however, diltiazem stimulated [^{3}H]PN200-110 binding in a dose-dependent manner as much as 40%. In this regard, diltiazem was also reported to have similar effects on DHP binding to brain and heart membranes (39, 40). Unlike in adrenal medulla membranes, however, diltiazem was able to stimulate DHP binding to skeletal muscle T-tubules even at 10° (41). At 37° diltiazem increased [^{3}H]PN200-110 binding by increasing the B_{max} of [^{3}H]PN200-110 binding without affecting the K_d (data not shown). Diltiazem was shown to increase DHP binding to heart, skeletal muscle, and smooth muscle membranes by a similar mechanism (8, 47, 50). In brain membranes, however, diltiazem stimulated DHP binding by decreasing the apparent dissociation constant (50, 58). It should be noted that at 37° the basal level of [^{3}H]PN200-110 binding to adrenal medulla membranes is decreased by 63%, as compared with that seen at 20°. The inclusion of diltiazem at 37° restores binding to near control level measured at 20°. Our results demonstrate the presence of an allosterically linked benzothiazepine receptor site in the adrenal gland, implying a common structural entity for these binding sites.

The calcium antagonist bepridil (4) has been shown to positively modulate the binding of DHPs to canine cardiac sarcolemma (8). Bepridil at low concentrations has been shown to stimulate the binding of [^{3}H]nitrendipine to membranes from intestinal smooth muscle (47), brain tissue (59), and uterine smooth muscle (60), whereas higher concentrations inhibit binding. The effect of bepridil in the bovine adrenal medulla is different, in that it only stimulated [^{3}H]PN200-110 binding, even at high concentrations (0.1 mM), which were reported to potently inhibit [^{3}H]nitrendipine binding to muscle membranes (8). These results indicate that the binding site for bepridil must also be present and linked to the DHP binding site on the Ca^{2+} channel in adrenal medulla membranes, although the negative allosteric effect is absent.

The dissociative anaesthetic PCP has recently been shown to inhibit release of catecholamines from whole adrenal glands and cultured adrenal cells (61, 62). PCP has also been shown to stimulate the binding of [^{3}H]nitrendipine to rat and mouse brain synaptosomal membranes (63). PCP mediated its stimulatory effect by reducing the apparent dissociation constant of [^{3}H]nitrendipine for its receptor (63). PCP was without significant effect on [^{3}H]PN200-110 binding even at 10 μM in adrenal medulla membranes. It appears, therefore, that the PCP binding site is not linked to the DHP site as in the brain. In this regard, PCP was also shown to have no effect on the binding of [^{3}H]nitrendipine to cardiac muscle membranes (63).

[^{3}H]Azidopine has been a powerful photoaffinity probe for identifying the DHP-binding subunit of the DHP receptor from muscle and brain membranes (5, 37). [^{3}H]Azidopine was specifically incorporated into a polypeptide of M_r 185,000–190,000 in adrenal medulla membranes, whereas the M_r 170,000 (α_1) subunit of the skeletal muscle DHP receptor was labeled under identical conditions. The apparent molecular weight of the [^{3}H]azidopine-labeled polypeptide in the adrenal medulla was approximately 10,000–15,000 larger than the DHP-binding polypeptide labeled in the skeletal muscle. The migration in SDS-PAGE of the DHP-binding polypeptide from the adrenal medulla was not altered by electrophoresis under reducing conditions (10 mM DTT), although the amount of radiolabel re-

covered was decreased (data not shown). A decrease in the amount of [^{3}H]azidopine detected when SDS-PAGE is carried out in the presence of DTT has previously been shown in skeletal muscle (29, 32). This phenomenon is due to a direct nucleophilic attack by DTT on the covalently attached [^{3}H] azidopine (37). These properties of the DHP-labeled polypeptide from adrenal medulla membranes are similar to the α_1 subunit of the DHP receptor-Ca^{2+} channel complex from skeletal muscle. The molecular weight of the DHP-binding polypeptide labeled in the adrenal medulla is similar to the molecular weight of the DHP-binding polypeptide covalently labeled in heart and brain (5, 35, 56). It appears that the molecular weight of the α_1 subunit in the adrenal medulla, heart, and brain is slightly larger than that in skeletal muscle (36, 37). This structural disparity may reflect fundamental functional differences in the role that DHP receptors play in these tissues. The fact that DHP receptors from the adrenal medulla could be immobilized and specifically eluted with sugars from WGA-Sepharose indicates a glycoprotein nature of the DHP-receptor complex. It has been reported that the α_1 DHP-binding subunit of the skeletal muscle Ca^{2+} channel is not glycosylated (35, 37) and that its retention on lectin columns is due to its association with the glycosylated α_2-subunit of the Ca^{2+} channel. If the DHP-binding polypeptide in the adrenal medulla is unglycosylated, our results provide circumstantial evidence for the presence of an α_2-like glycoprotein subunit, which would explain its retention on WGA-Sepharose. Confirmation of an α_2 subunit will require further studies. Elution of [^{3}H]azidopine-labeled adrenal medulla proteins from WGA-Sepharose required incubation in 0.5 M glucosamine for 2–3 hr. This is different from both cardiac and skeletal muscle DHP receptor, which can be eluted from the WGA-Sepharose column rapidly with low concentrations of glucosamine.[1] This suggests that the glycoprotein nature of the α_2-subunit or of the entire DHP receptor complex differs in the adrenal medulla, compared with muscle tissue.

In summary, our results demonstrate that the DHP binding sites in adrenal medulla membranes are part of a multidrug receptor complex that is probably associated with the L-type Ca^{2+} channel. Our results further demonstrate the presence of an α_1-like subunit of the L-type Ca^{2+} channel, which in the adrenal medulla may represent a subtype of DHP receptor Ca^{2+} channel.

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