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AN ANTIBODY TO DIHYDROPYRIDINE CALCIUM ENTRY BLOCKERS

A COMPARISON WITH THE CALCIUM CHANNEL RECEPTOR IN SKELETAL MUSCLE

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Abstract—Antibodies that recognize dihydropyridine (DHP) calcium entry blockers were elicited from rabbits. A sensitive and specific radioimmunoassay for dihydropyridines was developed and its specificity compared to the DHP binding site in skeletal muscle membranes. The antibody bound [^{3}H]nitrendipine with a higher affinity (K_D = 0.155 nM) than did the DHP receptor of skeletal muscle (K_D = 1–3 mM); however, in contrast to the DHP receptor, the antibody recognized only those DHP drugs with meta-nitrophenyl substituents at the 4-position on the DHP ring. Both the antibody and receptor exhibited stereospecificity, with each site recognizing the (+)-isomer of nicardipine as the more potent. This antibody should prove useful in our studies of some potentially irreversible DHP molecules.

The dihydropyridine (DHP‡) calcium channel antagonists are extremely useful both clinically [1] and as probes to investigate the structure and function of calcium channels, since their specific binding sites are thought to be associated with such channels [2]. The presence and levels of these drugs have been determined using a smooth muscle bioassay [2], a radioligand binding assay [3–5], and by high performance liquid chromatography [4]. The development of a radioimmunoassay for certain DHP drugs was undertaken in this laboratory, as part of an ongoing project searching for long-acting DHP calcium channel blocking drugs.

In the present study we describe the production of a rabbit anti-DHP antibody and its use in such a radioimmunoassay. This antibody bound [^{3}H]nitrendipine ([^{3}H]NIT) with high affinity, and comparisons were made of the abilities of several known and new DHP drugs to compete with [^{3}H]NIT in binding to either the antibody or to the DHP receptor of skeletal muscle. It was found that the antibody and the skeletal muscle DHP receptor exhibited similarities and also differences in their recognition patterns for DHP molecules.

MATERIALS AND METHODS

Materials. Bovine serum albumin and water-soluble carbodiimide were purchased from Sigma (St. Louis, MO). The sheep anti-rabbit antisera was obtained from Pel-Freez (Rogers, AK). Nitrobenzaldehyde and other organic reagents were obtained from Aldrich (Milwaukee, WI). [^{3}H]NIT, specific activity 77.4 Ci/mmole, was obtained from New England Nuclear (Boston, MA). The unlabeled DHP drugs were gifts from the following sources: felodipine, Hassle (Molndal, Sweden); CPG 28392, CIBA-Geigy (Basel, Switzerland); (+) and (−) nicardipine, Yamanouchi Chemical Co. (Japan) and NIT, nimodipine, nisoldipine and Bay k 8644 from Miles Pharmaceuticals (West Haven, CT). Nifedipine, its *m*- and *p*-nitro isomers and also its *m*-methyl isomer were synthesized in our UCI laboratory. Several as yet undescribed DHP compounds were synthesized by the Nelson Research & Development Co., of Irvine, CA, and are protected by that Company's patents.

Radioligand binding assay. [^{3}H]NIT binding assays have been described previously [6–7]. This DHP binding site on skeletal muscle T-tubules appears to be closely related to calcium channels and has been described as a receptor by some of these authors, a definition we shall use here to avoid confusion with the antibody binding site for DHP molecules. Briefly, 60–70 μg protein of skeletal muscle microsomes, prepared by the method of Fairhurst [8], were incubated for 30 min at 25° with 0.5 nM [^{3}H]NIT plus/minus inhibitory compounds in a final volume of 2 ml containing 12.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.4, and 1 mM $CaCl_2$. The specific activity of the [^{3}H]NIT was diluted 5-fold with unlabeled NIT to approximately 15 Ci/mmole. Membrane bound radioactivity was trapped at the end of the incubation of GF/B filters mounted on a model M-24R Brandel cell harvester. The filters were washed with 20 ml of ice-cold 12.5 mM HEPES buffer, pH 7.4, and counted in an

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‡ Abbreviations: DHP, dihydropyridine; NIT, nitrendipine; and HEPES, *n*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

LKB scintillation spectrometer which was calibrated to correct for quench and convert counts to disintegrations per minute. Binding in the presence of 10^{-6} M nifedipine was considered nonspecific. The use of this particular skeletal muscle membrane preparation in radiolabeled dihydropyridine binding experiments has not been reported, though the results are essentially the same as those obtained with a more purified preparation [9]. This membrane preparation was chosen for use in these assays because of the stability of the membranes to freezing, the relative ease of their preparation, and the good signal to noise ratio of the assay.

Preparation of the conjugate. The conjugate was produced by dissolving 12 mg of bovine serum albumin in 1.5 ml of water, and mixing this with 10 mg of N-0521 (see Table 2 for structure) dissolved in 3 ml of dimethylformamide and with 4 mg of water-soluble carbodiimide dissolved in 1.5 ml of water for 6 hr with constant stirring at room temperature in the dark. As the reaction proceeded, the pH of the mixture dropped from 9.4 to 5.2. When the reaction was complete, the mixture was dialyzed for 16 hr against dimethylformamide/water (1:1) followed by dialysis against 12.5 mM HEPES buffer, pH 7.4, for 16 hr. The buffer dialysis precipitated the conjugate which was lyophilized and stored at −20°.

Immunization. Albino New Zealand rabbits were inoculated intradermally at 12 sites on their backs with a total of 0.6 ml of an emulsion containing 1 vol. of the conjugate in water (2 mg/ml) mixed with 4 ml of Freund's complete adjuvant. The rabbits were injected intramuscularly in their hindquarters with 2 × 0.25 ml of the above conjugate emulsion 6 weeks after the primary inoculation. Animals were bled from the central ear artery periodically. All of the studies in this report were performed with antisera from a single bleeding 2 weeks after the first boost. Antisera were stored at 4° with 0.02% sodium azide.

Haptens. New compounds N-0490, N-0524, N-0520, N-0525, N-0546 and N-0548 (see Tables 1 and 2 for structures) were prepared according to a modified Hantzsch synthesis using the appropriate phthalimidoacetoacetic ester, benzaldehyde, and aminocrotonate. Removal of phthalimido from N-0490 and N-0520 generated the amines N-0494 and N-0521 respectively. Reaction of these amines with thiophosgene gave isothicyanates N-0494-NCS and N-0521-NCS. Pyrans A and B were prepared according to the literature [10, 11]. All of the compounds tested were made up as 1 mM solutions in ethanol and diluted with 1% ethanol to make 10-fold concentrated stock solutions. A final ethanol concentration of 0.1% was used in the assays, a concentration which did not affect either assay.

Antibody binding parameters. The immunological response was determined by measuring the binding of [^{3}H]NIT (1 nM) to antisera at a dilution of 1:1000. Dilutions were made with sample buffer which contained 140 mM NaCl, 10 mM $NaHPO_4$ (pH 7.0), 25 mM NaN_3, 10 mg/l phenol red and 0.1% (w/v) gelatin. The standard assay, composed of 0.2 ml of antisera (1:200), 0.2 ml of [^{3}H]NIT (10 mM), 0.2 ml of sample buffer and 0.2 ml of unlabeled hapten, was incubated for 16–24 hr at 4°. Then 0.2 ml of sheep anti-rabbit sera which had been preprecipitated with non-immune rabbit sera was diluted 1:5 with sample buffer, added to the incubation mixture, and incubated with shaking at 4° for 16–24 hr. The sheep anti-rabbit immunoprecipitate was prepared by mixing 173 ml of non-immune rabbit sera, that had been diluted 25-fold with modified sample buffer in which 50 mM EDTA and 10 mg/ml bromophenol blue were substituted for the gelatin and phenol red, with 100 ml of sheep anti-rabbit sera. The precipitate was allowed to form overnight, then centrifuged at 2000 *g* and finally resuspended in 53 ml of modified sample buffer. Aliquots were stored at −20°, snap thawed prior to use, and stored at 4° once thawed. The antibody-bound drug was separated from the free drug by filtration through GF/C filters mounted on a Brandel cell harvester followed by a 20-ml wash with ice-cold 12.5 mM HEPES buffer, pH 7.4. The filtration and wash were complete within 20 sec. Radioactivity in the immunoprecipitate trapped on the filters was determined by counting in 5 ml of liquiscint in an LKB scintillation spectrometer. Non-specific binding to sera and filters was defined as that bound in the presence of 10^{-6} M unlabeled NIT or that bound to non-immune sera; the results were equivalent.

Calculations. The average intrinsic association constant, K_0, and the concentration of antibody-combining sites, A_0, were determined by analysis of bound drug at various [^{3}H]NIT concentrations (0.015 to 1.5 nM). The data were plotted according to Scatchard [12] using a least squares linear regression to predict the x-intercept and the best curve through the experimental points. Data were corrected for non-specific binding, and free ligand was calculated by subtracting antibody-bound ligand from the total ligand added initially.

K_I values were calculated from IC_{50} values using the equation [13], $K_I = IC_{50}/(1 + F/K_D)$, where F is the free radioligand concentration and K_D is the dissociation constant for the antibody. The IC_{50} values were determined from Hill plot transformations of the data [14] and the K_D is the reciprocal of K_0. K_I and K_D values were determined by similar methods for the receptor assays.

RESULTS

Rabbits inoculated with a DHP–bovine serum albumin conjugate developed antibodies that bound [^{3}H]NIT with high affinity. The average intrinsic association constant, K_0, and the concentration of antibody-combining sites, A_0, were calculated from Scatchard plots. Experimental points are shown in Fig. 1, for the standard 1:1000 antisera dilution and [^{3}H]NIT concentrations ranging from 0.015 to 1.5 nM, with lines determined by least squares linear regression analysis ($r = -0.99$). In replicate determinations, K_0 was found to be equal to $6.45 \pm 0.54 \times 10^9$ liters/mole^{-1} and A_0 was equal to 356 ± 59 nM.

[^{3}H]NIT could be displaced from the antibody by unlabeled DHP haptens. In Fig. 2, unlabeled NIT is shown to displace the labeled drug. Plotting this data using a Hill plot transformation, not shown, produced a straight line with a correlation coefficient of 0.99 and a slope of 1.09. The IC_{50} determined from

Table 1. Comparison of the antibody and skeletal muscle binding sites for dihydropyridines

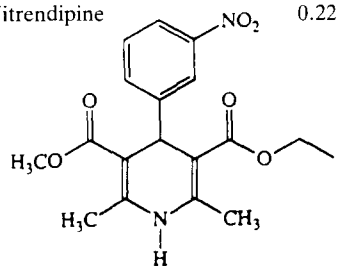
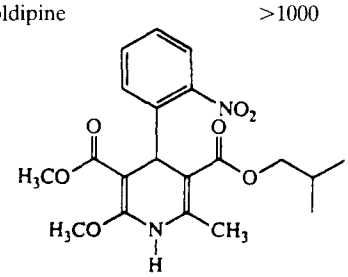
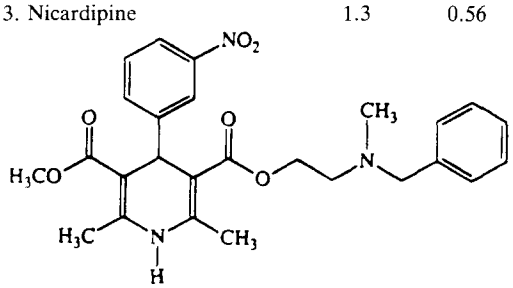
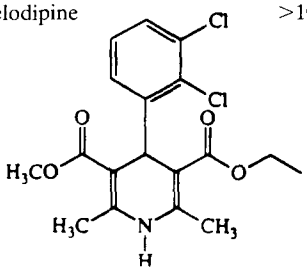
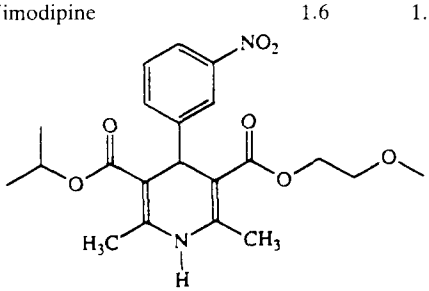
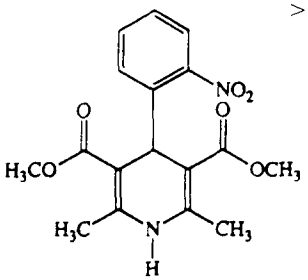
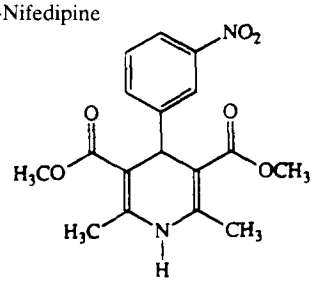
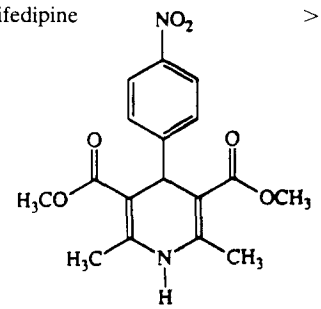
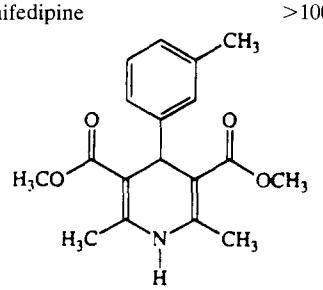
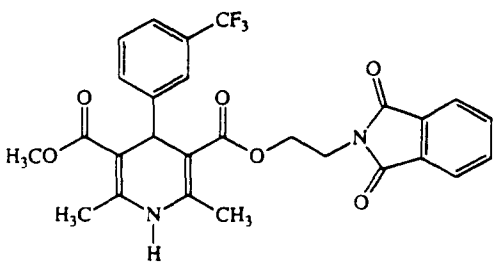
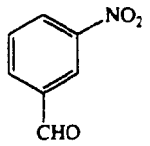
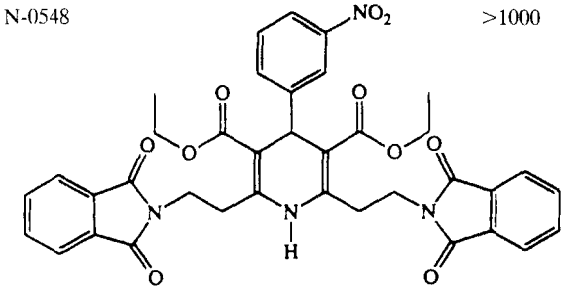
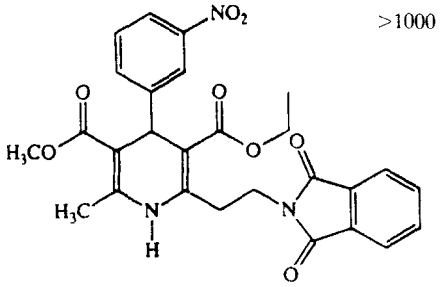
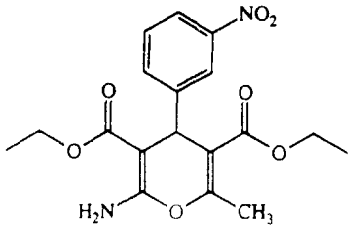
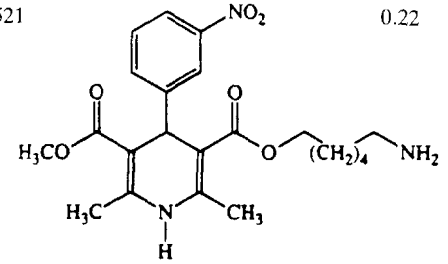
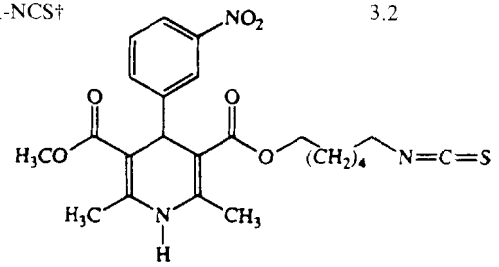
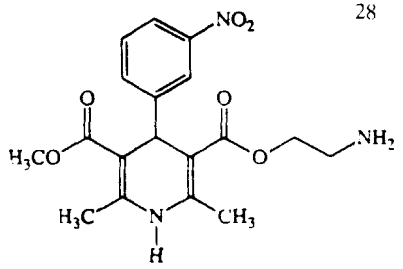
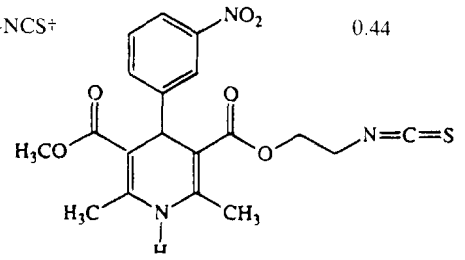
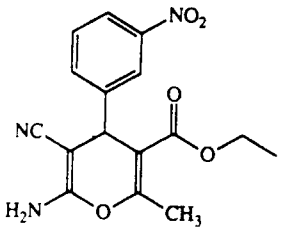
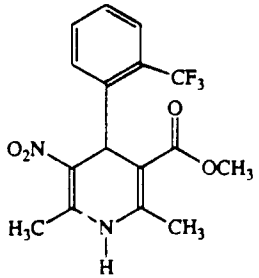
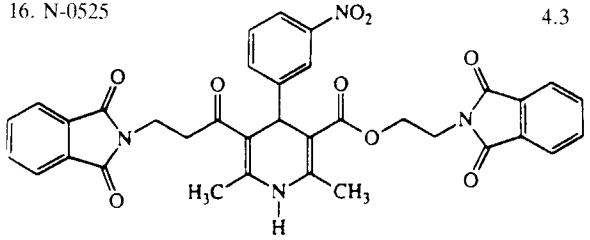
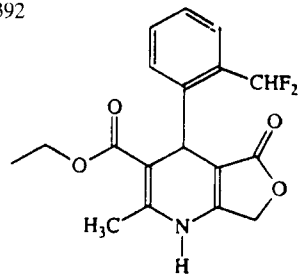
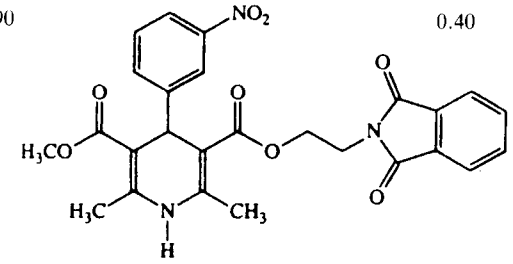
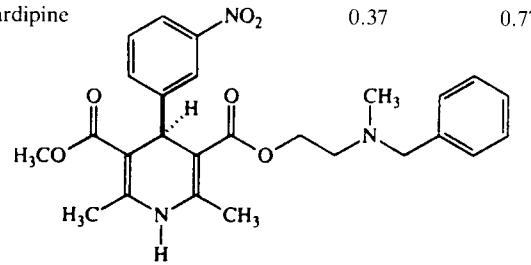
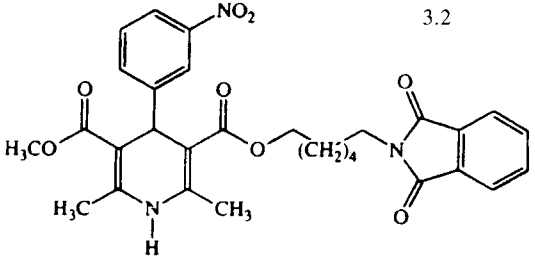
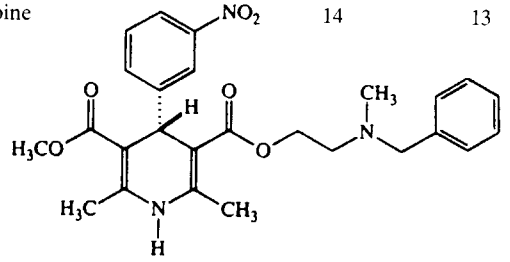
Name	Structure	K_I (nM) Antibody	Receptor
1. Nitrendipine		0.22	2.1
2. Nisoldipine		>1000	1.9
3. Nicardipine		1.3	0.56
4. Felodipine		>1000	1.3
5. Nimodipine		1.6	1.6
6. Nifedipine		>1000	20
7. *m*-NO_2-Nifedipine		4.0	14
8. *p*-NO_2-Nifedipine		>1000	>1000
9. *m*-Methyl-nifedipine		>1000	160
10. N-0524		>1000	48

Table 2. Comparison of the antibody and skeletal muscle binding sites for dihydropyridines

Name*	Structure	K_I (nM) Antibody	K_I (nM) Receptor
11. *m*-Nitrobenzaldehyde		>1000	>1000
12. N-0548		>1000	>1000
13. N-0546		>1000	200
14. Pyran A		28	68
19. N-0521		0.22	42
20. N-0521-NCS†		3.2	1.9
21. N-0494		28	>1000
22. N-0494-NCS†		0.44	3.0

15. Pyran B		97	>1000	23. Bay k 8644		>1000	68
16. N-0525		4.3	2.5	24. CGP28392		>1000	910
17. N-0490		0.40	0.70	25. (+)Nicardipine		0.37	0.77
18. N-0520		3.2	1.9	26. (−)Nicardipine		14	13

* The compound numbers continue from Table 1.

† The potentially irreversible nature of these compounds could make the calculation of K_I values by the Cheng–Prusoff equation inaccurate, though the equation was used for all the values presented here to simplify comparisons.

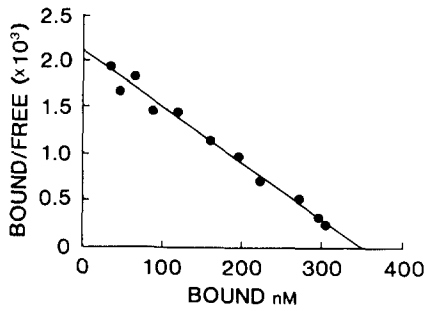


Fig. 1. Scatchard plot analysis for the determination of K_o and A_o of the binding of [^{3}H]NIT to the rabbit antibodies to DHP drugs. The assay was performed as described in Materials and Methods for [^{3}H]NIT concentrations ranging from 0.015 to 1.5 nM. The line was predicted by least squares linear regression analysis, $r = -0.99$.

this plot was 1.67 nM which, when converted to a K_i value, is 0.224 nM. This value is within reasonable agreement of the K_D value of 0.155 ± 0.013 nM determined by Scatchard plot analysis. The antibody has an approximately 10-fold greater affinity for [^{3}H]NIT than does the DHP receptor in skeletal muscle which we [9] and others [6, 7] have found to have a K_D of 1–3 nM. The sensitivity of this assay, $IC_{20} = 110$ pg/ml of assay volume, is comparable to DHP radioreceptor assays described previously [4, 5]. Also shown in Fig. 2 is the inability of the closely related drug nifedipine to displace [^{3}H]NIT from the antibody, illustrating the marked selectivity of the antibody.

To determine the nature of this specificity, the affinity of the antisera was determined for various DHP haptens and was compared to the ability of these haptens to inhibit [^{3}H]NIT binding to the DHP receptor of skeletal muscle. In Tables 1 and 2, the structures of various DHP molecules are shown along with the K_I values for the compounds determined in the antibody and the receptor assays.

Examination of the K_I values for the first six compounds shown in Table 1 indicate a marked contrast in affinity between the antibody and the receptor for these drugs. All six of these well-known DHPs bound to the receptor with nanomolar affinities, whereas only those with a meta nitro group on the phenyl ring were recognized by the antibody. Thus, NIT, nicardipine and nimodipine were bound by the antibody, whereas nisoldipine, felodipine and nifedipine were not. This point is particularly apparent when compounds 6 through 8 are examined. These compounds differ only in the placement of the nitro group; thus, it becomes obvious that only compound 7, with the meta placement, is recognized by the antibody. Neither the receptor nor the antibody recognized compound 8, with the para substitution, while compound 6 with the ortho placement, which is nifedipine, was bound to the receptor but not to the antibody. Furthermore, the requirement for a meta substituent is very specific for a nitro group, as indicated by the failure of the antibody to bind compounds 9 and 10 with meta methyl and trifluoromethyl moieties respectively. Both of these compounds were bound to the receptor, though neither was recognized by the antibody.

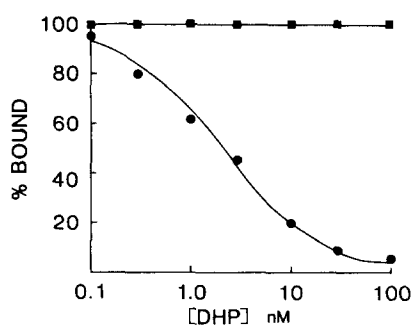


Fig. 2. Inhibition of [^{3}H]NIT binding to the rabbit antibody by unlabeled DHP drugs. Data represent the inhibition produced by nifedipine (■) or NIT (●).

To determine whether the antibody recognition site was limited to the phenyl ring, meta-nitrobenzaldehyde was tested and found inactive in both assays (Table 2). This result suggests that the DHP ring is required for antibody recognition of the molecule, and is confirmed by examination of compounds 12 through 15 where various substitutions on the DHP ring reduce both antibody and receptor affinity. It appears that both binding sites are very dependent on the steric properties of the DHP ring as opposed to electronic considerations, as indicated by the failure of either site to recognize compounds 12 and 13 with bulky substitutions, in contrast to the recognition of compound 14 in which the DHP ring has been converted to a pyran ring and the 6 methyl replaced with an amino group. These changes did reduce the affinity of both sites for this compound, however, and further modification by the replacement of an ester group with a cyano group reduced antibody affinity still further and resulted in complete loss of receptor recognition, as seen for compound 15. It is also interesting to note that interaction with neither the receptor nor the antibody was dependent on the size of the ester substitutions, as indicated by compounds 16 through 18.

Both the antibody and the receptor recognized the hapten, compound 19, which was conjugated to bovine serum albumin for the rabbit immunization, though the antibody showed a 100-fold greater affinity for the hapten than did the receptor. Furthermore, the potentially irreversible isothiocyanate derivative (compound 20) was also recognized in both assays. The similar compound, number 21, bound to both sites with a much lower affinity, suggesting that the placement of the amino substituent close to the DHP ring produces an impediment to the binding to either site. This idea is supported by the results with compound 22 in which the isothiocyanate derivative was approximately two orders of magnitude more potent at both sites.

The agonist compounds Bay k 8644 and CGP 28392 were both inhibitors of [^{3}H]NIT binding of the DHP receptor; however, since neither compound contains a meta nitro phenyl group, they were not recognized by the antibody. Both the antibody and the receptor exhibited stereospecificity, with each site recognizing the same enantiomer of nicardipine

as being the more potent. Thus, the DHP receptor bound the (+)-isomer of nicardipine (compound 25) with a 17 times greater affinity than the (−)-isomer. Similarly, the antibody recognized the (+)-isomer as the more potent, with a eudismic ratio of 38.

DISCUSSION

This report describes a rabbit antibody that recognizes some, although not all, of the DHP calcium entry blocking drugs tested which bind to the DHP receptor of skeletal muscle. This antibody was raised against a DHP–bovine serum albumin conjugate and was found to recognize only those DHP haptens which, like the antigen (N-0521), contained meta nitrophenyl substituents at the 4 position on the DHP ring. Thus, it does not recognize the clinically useful drug, nifedipine, which has an ortho nitrophenyl substituent. From the results of this study, however, it seems likely that a radioimmunoassay for nifedipine could be developed easily, provided that an ortho nitrophenyl substituted DHP was used as an antigen. Obviously, a radioligand structurally similar to the antigen would also be required since the antibody recognition site can detect subtle structural differences, as exemplified in this study by the ability of the antibody to recognize only those compounds with ortho nitrophenyl groups.

The present antibody has a high affinity for the DHP drugs nimodipine and nicardipine and exhibits a 10-fold higher affinity for [^{3}H]NIT than does the DHP receptor of skeletal muscle. This antibody can thus be employed in a radioimmunoassay for selected DHP drugs and is capable of detecting 110 pg of NIT in the 1 ml assay volume. This sensitivity compares favorably with previously reported radioreceptor assays for DHP drugs [4, 5]. The sensitivity of the present assay can be enhanced by decreasing the [^{3}H]NIT concentration in the assay; however, the unique selectivity of the antibody is the focus of this report. Aside from the specific requirement for a meta nitrophenyl substituent for antibody recognition, both the antibody and the receptor were very similar in other respects. Neither the antibody nor the DHP receptor was sensitive to the size of the 3.5 ester substituents, though the ester was required for high affinity recognition. Modification at positions 1, 2 or 6 on the DHP ring greatly reduced the ability of the compounds to bind to either the antibody or the receptor, particularly if large substitutions were made. The antibody appeared to be less sensitive to the electronic character of the substitutions than did the receptor, exhibiting a smaller decrease in affinity for molecules with polar amino groups in close proximity to the DHP ring. Both the antibody and the receptor recognized the same (+)-enantiomer of nicardipine as being the more potent.

These results thus describe some aspects of the recognition patterns of this antibody and of the skeletal muscle DHP receptor for a series of DHP drugs. This antibody should prove useful in our studies of the potentially irreversible isothiocyanate DHP molecules, such as compounds 20 and 22.

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