Effect of Neuraminidase on Diltiazem-Mediated Alteration of Nitrendipine Binding in the Hog Coronary Artery

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Abstract—The dissociation constant (Ka) and the maximum number of binding sites (Bmax) of 3H-nitrendipine (3H-NTD) were not altered by neuraminidase (NUase) treatment. The Ka and Bmax values were approximately 0.2 nM and 70 fmoles/mg protein, respectively. The influences of diltiazem on 3H-NTD bindings differed between NUase treated and untreated preparations. Diltiazem increased 3H-NTD (100 pM) binding dose-dependently with a 40% increase at about 10^-6 M in the untreated preparation. After treatment with NUase, a low dose of diltiazem decreased 3H-NTD (100 pM) binding, and the greatest effect was observed at 10^-9 M with a 50% decrease, while at a high dose of diltiazem (10^-6 M), 80% increase of the binding was observed. Scatchard plot analysis indicated that a high dose of diltiazem (10^-6 M) increased only the affinity of 3H-NTD to the binding site in both the NUase-treated and untreated preparations. In the untreated preparation, a low dose of diltiazem (10^-9 M) had no effect on 3H-NTD binding, but in the NUase-treated preparation, diltiazem (10^-9 M) showed opposing effects, namely, an increase and decrease of the binding at low and high concentrations of 3H-NTD, respectively. No straight line was obtained when a hill plot analysis of the effect of diltiazem on 3H-NTD binding was made. These results suggested that diltiazem allosterically regulates dihydropyridine derivative binding, and in this regulation, the concentration ratios between diltiazem and the dihydropyridine derivative is the most important factor. Furthermore, it is suggested that the sialic acid of the proteoglycan is involved in these interactions, especially in the effect of diltiazem on dihydropyridine binding.

The negative inotropic action of calcium entry blockers (calcium (Ca)-antagonists) is considered to be due to the specific blockage of the slow channel (1–3). Pharmacological and electrophysiological studies on the Ca-antagonists suggest that structurally dissimilar antagonists affect the slow calcium channel through different modes of action (4, 5). Recently, 1, 4 dihydropyridine derivatives such as 3H-nitrendipine (3H-NTD), 3H-nimodipine and 3H-nifedipine have been used to label the calcium channel in brain, heart and coronary artery preparations (6–12). An allosteric interaction may occur among the binding sites of structurally dissimilar Ca-antagonists such as 1,4 dihydropyridine, phenylalkylamines and a benzothiazepine derivative (diltiazem) (8, 10, 13, 14). Diltiazem stimulates 3H-labeled 1,4 dihydropyridine binding to cardiac, skeletal muscle and brain by increasing the number of binding sites or increasing the affinity to the binding sites (13–15). The involvement of this binding in the pharmacological actions of Ca-antagonists also has been reported (15). Recently, Curtis and Catterall (16) speculated that the Ca-antagonist binding sites have a glycoprotein containing N-acetylglucosamine and/or sialic acid residue. In addition, it is well known that the sarcolemma of cardiac muscle has an glycocalyx that consists of a surface coat and an external lamina. The former layer is an integral part of the sarcolemma and many of
its glycoproteins penetrate into or through the lipid layer (17). The terminal monosaccharide unit in the glycoprotein is often a negatively charged residue of N-acetyl-neuraminic acid, a sialic acid (17). In this study, we investigated the involvement of N-acetyl-glucosamine and/or sialic acid in the binding of Ca-antagonists and the interaction between the binding sites of Ca-antagonists in the coronary artery of the hog.

Materials and Methods

The membrane fraction of hog coronary artery was prepared according to the method of DePover et al. (10). Hog hearts were obtained from a local slaughterhouse. Soon after the animals were sacrificed, the fresh hearts were placed in ice-cold Tyrode solution and immediately transported to the laboratory. The left anterior descending arteries and the right circumflex arteries were dissected, opened longitudinally, minced with scissors and then homogenized with a glass homogenizer in 10 vol. of ice-cold 50 mM Tris-HCl (pH 7.5). The homogenate was filtered through silicon-coated gauze. The filtrate was centrifuged at 500xg for 10 min, and the supernatant was then centrifuged at 12,000xg for 10 min. The resulting supernatant was centrifuged at 105,000xg for 30 min. The pellet was resuspended in 50 mM Tris-HCl (pH 7.5) and used as the coronary artery preparation; the concentration of this preparation was 0.3 to 0.5 mg original tissue per ml.

In some experiments, the final pellet was treated with neuraminidase (NUase) according to the method of Nagatomo et al. (18) using medium containing 100 mM KCl, 10 mM MgCl₂, 20 mM Tris-maleate (pH 7.4) at 25°C for 30 min. Following the incubation, the preparation was centrifuged at 105,000xg for 30 min. The pellet was resuspended in 50 mM Tris-HCl (pH 7.5) and used as the coronary artery preparation; the concentration of this preparation was 0.3 to 0.5 mg original tissue per ml.

For the binding assay, 3H-NTD was incubated at 23°C for 30 min with the suspended preparation at a final volume of 2 ml containing 50 mM Tris-HCl (pH 7.5). Membrane bound 3H-NTD in the incubation mixture was trapped at the end of the incubation by rapid vacuum filtration through a Whatman glass filter (GF/B). The filters were rinsed with 5 ml of ice-cold 50 mM Tris-HCl (pH 7.5). The radioactivity of the filter was measured using a liquid scintillation spectrometer. We defined 3H-NTD binding in the presence of 1 μM nifedipine as the non-specific binding.

Kinetic analysis of non-linear Scatchard plots was carried out on a computer system (19) based on the method of Feldman (20) using a PC-8001 (NEC) personal computer. Each assay was carried out in duplicate.

The protein concentration was determined using bovine serum albumin as the standard (21).

Nifedipine and Diltiazem were prepared in our laboratory from Adalat (Bayer) and Herbesser (Tanabe), respectively. NUase and 3H-NTD (S.A.: 70 Ci/mmol) were obtained from Sigma and New England Nuclear, respectively. All other chemicals were obtained from commercial sources.

Results

NUase treatment significantly decreased the sialic acid content in the coronary artery from 3.27±1.40 nmoles/mg protein to 0.80±0.64 nmoles/mg protein, but did not alter the protein content. 3H-NTD binding to the coronary artery was not affected by the NUase treatment, while the effect of diltiazem on the binding was significantly altered (Fig. 1). In the untreated preparation, diltiazem increased the 3H-NTD binding in a dose-dependent manner, with the maximum effect (40%) being observed at a concentration of 10⁻⁶ M. In the NUase-treated preparation, however, diltiazem decreased the 3H-NTD binding at doses lower than 10⁻⁸ M and increased it at doses higher than 3x10⁻⁷ M. The greatest effects were observed at 10⁻⁹ M and 10⁻⁶ M, which caused a 50% decrease and 80% increase, respectively.

Scatchard plot analysis of 3H-NTD binding indicated that 10⁻⁹ M diltiazem only increased the affinity of 3H-NTD to the binding site in both the NUase-treated and untreated preparations (Fig. 2). At low doses, however, the effects of diltiazem on the 3H-NTD binding depended on whether the pre-
In the untreated preparation, $10^{-9}$ M diltiazem had no effect on the binding of $^3$H-NTD (Fig. 3b), while in the NUase-treated preparation, Scatchard plot analysis of the $^3$H-NTD binding in the presence of $10^{-9}$ M diltiazem showed the existence of two binding sites in the coronary artery (Fig. 3a). Diltiazem had an opposing effect on the $^3$H-NTD binding. At a low dose (1 nM), diltiazem decreased the $^3$H-NTD binding when the concentration of $^3$H-NTD was higher than 100 pM, but increased the binding when the concentration was lower than 50 pM (Fig. 3a). This shows that the coronary artery has two different affinity binding sites to $^3$H-NTD in the presence of $10^{-9}$ M diltiazem in the NUase-treated preparation. These opposing effects of diltiazem on $^3$H-NTD binding in the Scatchard plot corresponded to the two different effects of diltiazem which

### Table 1: Scatchard Plot Analysis of $^3$H-NTD Binding

| Condition          | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) |
|--------------------|------------|----------------------------|
| None               | 0.22 ± 0.06| 77.3 ± 4.6                 |
| Diltiazem (1μM)    | 0.13 ± 0.02| 76.9 ± 8.8                 |

### Table 2: Scatchard Plot Analysis of $^3$H-NTD Binding

| Condition          | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) |
|--------------------|------------|----------------------------|
| None               | 0.23 ± 0.04| 64.8 ± 10.3                |
| Diltiazem (1μM)    | 0.16 ± 0.03| 63.7 ± 7.0                 |

Fig. 1. Effect of NUase treatment on diltiazem-mediated $^3$H-NTD binding in the hog coronary artery. Control values of $^3$H-NTD binding in NUase treated and untreated preparations were 18.6±1.8 and 21.3±3.7 fmol/mg prot., respectively, when 0.1 nM $^3$H-NTD was used as a ligand. The values were obtained from 3 separate experiments.

Fig. 2. Scatchard plot analysis of $^3$H-NTD binding in the presence and absence of 1 μM diltiazem in the NUase treated (b) and untreated (a) hog coronary artery preparations. The values were obtained from 3 separate experiments.
were seen in the displacement experiment of $^3$H-NTD binding by diltiazem (Fig. 1.). These results clearly indicate that the effects of diltiazem depend on the concentration of $^3$H-NTD.

The different influences of diltiazem on the $^3$H-NTD binding, which depended on the concentration of $^3$H-NTD, were observed in the NUase-treated preparations (Fig. 4). When 100 pM of $^3$H-NTD was used as the ligand, diltiazem showed two opposing effects, decrease and increase of $^3$H-NTD binding, while when 25 pM of $^3$H-NTD was used, diltiazem only increased the binding. The dose-response curve of the effect of diltiazem on $^3$H-NTD binding shifted to the right and the potentiating rate of $^3$H-NTD binding was significantly increased. Maximum increases were observed at $10^{-6}$ M and $10^{-7}$ M of diltiazem when the concentrations of $^3$H-NTD were 100 pM and 25 pM, respectively. The lines obtained from the Hill plots were not linear with either concentration of $^3$H-NTD (Fig. 5). In the untreated pre-

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**Fig. 3.** Scatchard analysis of $^3$H-NTD binding in the presence and absence of 1 nM diltiazem in the NUase treated (b) and untreated (a) hog coronary artery preparations. The values were obtained from 3 separate experiments.

**Fig. 4.** Influence of $^3$H-NTD concentration on diltiazem-mediated $^3$H-NTD binding in the hog coronary artery. Control values of $^3$H-NTD binding when 25 and 100 pM were used were 10.3±2.7 and 18.6±1.8 fmoles/mg prot., respectively. The values were obtained from 3 separate experiments.
Preparations, the effect of diltiazem on $^3$H-NTD binding was also non-linear in the Hill plot, but the slope of the curve was more gentle than that in the NUase-treated preparations.

Discussion

The results obtained in this study indicate that NUase treatment of microsomal preparations of the coronary artery has no effect on the binding of NTD, but alters the influence of diltiazem on the NTD binding. Numerous workers have reported that the affinity of $^3$H-labeled dihydropyridines may be related to the pharmacological potency of the compound in smooth and cardiac muscles (9, 10, 15, 22). The stimulatory effects of diltiazem on $^3$H-nimodipine binding were also observed in smooth muscle and cardiac muscles (15). The mechanism of this interaction was considered to be the allosteric effect of diltiazem on the dissociation constant of dihydropyridine binding (9, 13-15).

Polar groups of the phospholipids, sulfated glycoproteins, and glycosaminoglycans contribute to the negative charges on the membrane surface of cells of the cardiovascular system (16). Ca, not K, exchange in the myocardial cell was strikingly altered by the treatment with NUase (16). Such observations suggest that sialic acid, which is cleaved from the O-glycosidic link by NUase, plays an important role in the maintenance of membrane selectivity for Ca (16). Recently, Curtis and Catterall (17) succeeded in the solubilization of the binding site of $^3$H-NTD and reported that the binding site was a glycoprotein-containing N-acetylglucosamine and/or sialic acid. Concerning the interaction between the binding sites of 1,4 dihydropyridines and other Ca-antagonists, they observed that the rate of dissociation of $^3$H-NTD was decreased by diltiazem and increased by verapamil in solubilized membranes, as was seen with non-solubilized membrane preparations. The binding site for the dihydropyridines is the second binding site coupled allosterically for the Ca-antagonists verapamil and diltiazem. From these results, they suggested that these Ca-antagonist binding sites are located on the same molecular complex that was a glycoprotein-containing N-acetyl-glucosamine and/or sialic acid.
Concerning the involvement of sialic acid in the alteration of the membrane surface, Nagatomo et al. (18) reported that NUase treatment of the crude cardiac plasma membrane (sarcolemma) fraction increased the binding sites for a β-adrenergic antagonist, 3H-dihydroalprenolol, with good reproducibility. From these results, they suggested that the removal of sialic acid from the glycocalyx of the sarcolemma induced an alteration of the environment of the receptor sites and improved the reproducibility of the binding.

The present results indicate that the binding of 3H-NTD was not affected by sialic acid removal by NUase treatment, but the effect of diltiazem on 3H-NTD binding was significantly altered. Furthermore, our results show that the concentration ratio between dihydropyridine and diltiazem is an important factor for the effect of diltiazem on dihydropyridine binding.

Taking these data together, it can be considered that the negatively charged residue sialic acid has an important role in the recognition of the information at the membrane surface.

In conclusion, the present results suggest that sialic acid is involved in the interaction between the binding sites of dihydropyridine and diltiazem, especially affecting the effective site of diltiazem on which diltiazem regulates the binding of dihydropyridine.

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ERRATUM

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The authors wish to point out the following errors:

- page 219, legend for Fig. 2. (b) should read (a), and (a) should read (b)
- page 220, legend for Fig. 3, (b) should read (a), and (a) should read (b)