Effects of Guanosine 5'‐Triphosphate on the Specificity of Irreversible α₁‐Antagonisms by Phenoxybenzamine in Rabbit Thoracic Aorta

Noriko Kokubu, Mitsutoshi Satoh and Issei Takayanagi *

Department of Chemical Pharmacology, Toho University School of Pharmaceutical Sciences,
Miyama 2-2-1, Funabashi, Chiba 274, Japan

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ABSTRACT — Phenylephrine displacement curves for the specific binding of [3H]prazosin in the membrane fraction prepared from rabbit thoracic aorta showed high- and low-affinity sites with slope factors significantly less than unity. The irreversible α₁-B-antagonist phenoxybenzamine shifted the binding sites to single high affinity sites with a slope factor close to unity in the presence of the metabolically stable GTP analog GTPγ-S. These results indicate that phenoxybenzamine may have affected selectively the low affinity site to phenylephrine in the presence of GTPγ-S.

Keywords: Phenoxybenzamine, α₁-Adrenoceptor subtype, G protein

α₁-Adrenoceptors belong to the family of membrane-spanning receptors that are coupled to guanine nucleotide-binding proteins (G proteins). α₁-Adrenoceptors have been further subclassified into α₁A-receptors and α₁B-receptors in the smooth muscle cells (1, 2). Phenoxybenzamine has been noted as a non-selective irreversible α-antagonist. Takayanagi and co-workers, however, have recently reported that phenoxybenzamine interacts more selectively with the α₁B-adrenoceptor subtype than with the α₁A-adrenoceptor subtype in rabbit thoracic aorta and common iliac artery (3).

Takayanagi et al. (4) proposed that in intestinal smooth muscles, there are two types of muscarinic receptors, one is sensitive to and the other is resistant to propylbenzilycholine mustard (PrBCM). Kiuchi et al. (5) reported that PrBCM discriminates between PrBCM-sensitive and -resistant muscarinic receptors in the presence of GTP but not in its absence. Both phenoxybenzamine and PrBCM are β-chloroethylamine derivatives. Therefore, similar mechanisms are considered to be involved in the discrimination between α₁A- and α₁B-adrenoceptors. In this paper, we tested whether or not guanine nucleotides are necessary to recognize two α₁-adrenoceptors, α₁A* and α₁B-subtypes.

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The thoracic aortae were dissected out from male albino rabbits weighing 2.0–3.0 kg. Endothelial cells were removed by gentle rubbings of the luminal surface with a cotton probe. The aorta was incubated in 95% O₂ / 5% CO₂ gassed Krebs-Henseleit solution. The composition of this buffer solution was: 118 mM NaCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 25.0 mM NaHCO₃ and 11.0 mM glucose dissolved in distilled water (pH = 7.4 at 37°C). To determine the effects of phenoxybenzamine, an irreversible antagonist of α₁-adrenoceptors, the tissues were treated with phenoxybenzamine (10⁻⁷ M) for 15 min.

The membrane preparations were obtained from the tissues by the method described previously (6). The [³H]prazosin binding assay was performed using membrane preparations in 50 mM Tris-HCl, according to the method used by Takayanagi et al. (7). The membrane preparations (150 μg protein/tube) were incubated with 0.2 nM [³H]prazosin in the presence or absence of 10 μM GTPγ-S in 50 mM Tris-HCl buffer for 30 min at 32°C. Aliquots were rapidly filtered through Whatman GF/C filters, and the filters were washed with 3 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4). After the passage, the filter was dried and radioactivity was determined in a toluene base scintillator with a liquid scintillator spectrometer (Aloka LSC-900). Nonspecific binding of [³H]prazosin was determined as the radioactivity

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*To whom correspondence should be addressed.
bound to membranes which was not displaced by 10 μM phentolamine. Specific binding was determined as the total radioactivity minus the nonspecific binding. All assays were conducted in duplicate. Data obtained from competition studies were analyzed by the weighted least-squares iterative curve fitting program LIGAND (8). The inhibition constant (K_i) was calculated by the equation of Cheng and Prusoff (9). The data were first fitted to a one- and then a two-site model, and if the residual sums of the squares were statistically less for a two-site fit of the data than for a one-site fit, as determined by an F-test comparison, then the two-site model was accepted.

Numerical results are expressed as means ± S.E., and statistical significance was calculated by Student’s t-test or Duncan’s new multiple range test. A P value less than 0.05 was considered to indicate a significant difference.

Drugs used were phenylephrine hydrochloride (Wako-Junyaku, Osaka, Japan), phenoxybenzamine hydrochloride (Tokyo Kasei, Tokyo, Japan), and [3H]-prazosin (specific activity 82 Ci/mmol, NEN, Boston, USA). Other chemicals used were of analytical grade.

Specific binding of [3H]prazosin (0.01–1.0 nM) in the membrane preparations was saturable, forming a plateau between 0.5 to 0.6 nM [3H]prazosin. The Scatchard plot was linear, suggesting a single population of binding sites with a K_D value of 0.17 ± 0.02 nM. The inhibition curves for the agonist phenylephrine are shown in Fig. 1A. The Hill coefficient for the drug obtained from the Hill plot (Fig. 1 inset) was 0.28 ± 0.04 (n = 6) for phenylephrine. This agent interacted with both high- and low-affinity sites labeled by [3H]-prazosin. The K_i values obtained by computer analysis of the competition curves are shown in Table 1. Pretreatment of the tissue containing sufficient activating factors, such as GTP, with 0.1 μM phenoxybenzamine increased the Hill coefficient and eliminated the low affinity site of the displacement curve (Fig. 1A). However, the same pretreatment of tissues that did not contain GTP had no effect on the displacement curve and the Hill coefficient was the same as that estimated in the untreated preparations (Fig. 1B). In the membrane preparation, the GTP analog GTPγ-S (10 μM) did not affect the total number of [3H]prazosin binding sites, and its Hill coefficient for the displacement curve was different from unity. However, the low affinity site of the curve was completely eliminated with phenoxybenzamine treatment in GTPγ-S containing membrane preparations (Fig. 1C, Table 1). Furthermore, addition of the ATP analog ATPγ-S (100 μM) and GDP analog GDPβ-S (30 μM) to the membrane preparations did not affect the displacement curves obtained from

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phenylephrine. In addition, GDP/3-S (30 µM) inhibited the effect of GTPy-S (10 µM) on the displacement curves obtained from phenylephrine (Table 1).

α₁-Adrenoceptors in membranes prepared from rabbit thoracic aorta were selectively labeled by [3H]prazosin, and then the labeled receptors were displaced by the α₁-agonist phenylephrine. The computer analysis of the displacement curve showed both high-affinity and low-affinity sites. The low-affinity sites were eliminated by the phenoxybenzamine pretreatment of the tissue preparation, and the displacement curves obtained from the pretreated membrane did not differ from those obtained from the untreated preparations. However, in membrane preparations in the presence of GTPy-S, low-affinity sites were completely eliminated by phenoxybenzamine. These results show that phenylephrine distinguished at least two different α₁-adrenoceptor binding sites in the membrane preparations from rabbit thoracic aorta.

Piascik et al. (10) reported that phenylephrine interacted with high- and low-affinity sites labeled by [3H]prazosin, and that the α₁-agonist phenylephrine displaced the α₁-agonist phenylephrine. The computer analysis of the displacement curve showed both high-affinity and low-affinity sites. The low-affinity sites were eliminated by the phenoxybenzamine pretreatment of the tissue preparation, and the displacement curves obtained from the pretreated membrane did not differ from those obtained from the untreated preparations. However, in membrane preparations in the presence of GTPy-S, low-affinity sites were completely eliminated by phenoxybenzamine pretreatment. These results show that phenylephrine distinguished at least two different α₁-adrenoceptor binding sites in the membrane preparations from rabbit thoracic aorta. In the present study, in the tissue and 10⁻⁵ M GTPy-S containing membrane fraction derived from the rabbit thoracic aorta, pretreatment with 10⁻⁷ M phenoxybenzamine decreased the [3H]prazosin specifically bound to approximately 28% and 35% of the control determined in the absence of phenoxybenzamine. However, the same treatment of the membrane preparation not containing GTPy-S did not change the amount of specific binding so that it remained the same as that of the untreated preparation. As it has been demonstrated in the tissue and the GTPy-S-containing membrane fraction, the phenoxybenzamine-sensitive α₁-subtypes in the rabbit thoracic aorta are likewise α₁B-adrenoceptor subtypes, and the phenoxybenzamine-resistant subtypes are α₁A-receptors. These findings indicate that phenoxybenzamine may interact more selectively with the α₁B-subtype in the presence of GTP or its analog but not in the absence of either of them, and suggest that GTP-related regulatory proteins intracellularly modulated the affinities or properties of α₁-adrenoceptors. Furthermore, the present results were similar to the findings of Kiuchi et al. (5) in the guinea pig intestinal smooth muscles where the irreversible antagonist PrBCM recognized two distinct population or states of muscarinic receptor sites in the presence of guanosine 5’-triphosphate (GTP).

### Table 1. Effects of GTPγ-S, ATPγ-S and GDPβ-S on irreversible α₁-antagonism by phenoxybenzamine for [3H]prazosin binding

|                | n   | pIC₅₀       | nH     | H       | L       | pKᵢ | %   | pKᵢ | %   |
|----------------|-----|-------------|--------|---------|---------|------|-----|------|-----|
| Control        | 6   | 5.14 ± 0.15 | 0.28 ± 0.04* | 5.55 ± 0.22 | 20.48 ± 2.52 | 4.46 ± 0.18 | 78.38 ± 2.22 |
| Tissue         |     |             |        |         |         |      |     |      |     |
| PBZ            | 3   | 5.10 ± 0.19 | 1.04 ± 0.02 | 5.55 ± 0.19 | 100    |      |     |      |     |
| Membrane       |     |             |        |         |         |      |     |      |     |
| PBZ            | 3   | 5.15 ± 0.18 | 0.27 ± 0.01* | 5.60 ± 0.11 | 17.17 ± 5.77 | 4.18 ± 0.16 | 82.66 ± 5.72 |
| PBZ + GTPγ-S   | 3   | 4.90 ± 0.06 | 1.05 ± 0.14 | 5.30 ± 0.07 | 100    |      |     |      |     |
| PBZ + ATPγ-S   | 3   | 5.48 ± 0.32 | 0.29 ± 0.02* | 5.72 ± 0.14 | 19.03 ± 6.39 | 4.48 ± 0.09 | 80.97 ± 6.39 |
| PBZ + GDPβ-S   | 3   | 5.48 ± 0.25 | 0.26 ± 0.02* | 5.92 ± 0.25 | 18.09 ± 2.89 | 4.55 ± 0.22 | 81.91 ± 1.48 |
| PBZ + GTPγ-S + GDPβ-S | 3 | 5.43 ± 0.06 | 0.26 ± 0.01* | 5.87 ± 0.06 | 11.95 ± 0.72 | 4.56 ± 0.04 | 88.05 ± 0.72 |

The membrane was incubated with 0.2 nM [3H]prazosin, and each concentration of phenoxyphrine. pKi, negative logarithm of the Kᵢ value. The IC₅₀ value was the molar concentration of unlabeled drug necessary to displace 50% of the specific binding. The populations of binding sites with high and low affinity are designated as percentages. n, number of experiments; nH, pseudo Hill coefficient; PBZ, phenoxybenzamine treatment. The values represent means ± S.E. * significantly different from unity (P < 0.05).
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