Characterization of $\beta$-Adrenoceptors in Pig Basilar Artery from Functional and Radioligand Binding Studies

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ABSTRACT—$\beta$-Adrenoceptors in pig basilar arteries were investigated by measuring the relaxation responses to norepinephrine and by a radioligand binding assay with $[^3H]$-dihydroalprenolol (DHA). Norepinephrine induced concentration-dependent relaxations. The relaxation responses were independent of the presence of endothelial cells, and they were competitively antagonized by ($\pm$)-propranolol, atenolol, butoxamine and ICI 118,551. Specific $[^3H]$-DHA binding to $\beta$-adrenoceptors was saturable, reversible and high affinity ($K_d=1.4 \text{ nM}$), with a $B_{\text{max}}$ of 48.7 fmol/mg protein. Computer analysis of inhibition of $[^3H]$-DHA binding by atenolol, butoxamine and ICI 118,551 gave a $\beta_1:\beta_2$-adrenoceptor ratio of approximately 65:35. The pA$_2$ values of these antagonists were significantly correlated with the $K_i$ values for $\beta_1$-adrenoceptor determined by the radioligand binding assay. The present findings indicate that the relaxation responses to norepinephrine are predominantly mediated through the stimulation of $\beta_1$-adrenoceptors on vascular smooth muscle cells in a pig basilar artery.

Keywords: Basilar artery (pig), Relaxation, $\beta_1$-Adrenoceptor, Norepinephrine, $[^3H]$-Dihydroalprenolol

Species differences have been reported concerning the responsiveness of basilar arteries to norepinephrine in vitro. Basilar arteries from monkeys (1), dogs (2), guinea pigs (3) and rabbits (4) respond to norepinephrine with contractions, and a rat basilar artery has no response (3), whereas, bovine (5) and pig (6) basilar arteries respond with relaxations, which are mediated through the stimulation of $\beta$-adrenoceptors. However, the $\beta$-adrenoceptor subtype responsible for the relaxation of pig basilar arteries has not been determined.

The present study was undertaken to clarify the distribution of $\beta$-adrenoceptor subtypes and the functional roles of these receptors in pig basilar artery, by measuring the effects of $\beta_1$- and $\beta_2$-antagonists on both the norepinephrine-induced relaxation response and the specific $[^3H]$-dihydroalprenolol (DHA) binding to membrane fractions.

MATERIALS AND METHODS

Basilar arteries from freshly slaughtered pigs were obtained at a local slaughterhouse and transferred to our laboratory immersed in ice-cold physiological salt solution (119 mM NaCl, 4.7 mM KCl, 1.6 mM CaCl$_2$, 1.2 mM MgCl$_2$, 25 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$ and 10 mM glucose) aerated with a mixture of 95% O$_2$ and 5% CO$_2$. The basilar artery was dissected free and cleaned of adhering tissues, and two rings about 4 mm in length were cut. A ring was mounted vertically between two L-shaped stainless steel holders fixing the upper region to an isometric force transducer (TB-611T, Nihon Kohden Kogyo Co.) and suspended in a 15 ml water-jacketed organ bath with oxygenated salt solution at 37°C (pH 7.4). Rings (outer diameter: 0.5–0.9 mm) mounted in the organ bath were left to equilibrate for at least 120 min under the resting tension of 0.75 g, which was optimal for inducing the maximal contraction. KCl (60 mM) solution was applied every 30 min until the amplitude of the contraction reached a constant value. Changes in KCl concentration in the physiological salt solution were compensated for by an equimolar adjustment of the NaCl concentration. The isometric tension development was displayed on an ink-writing recorder (WI-641G, Nihon Kohden Kogyo Co.). Cumulative concentration-response curves for norepinephrine were obtained by adding norepinephrine solution directly to the bathing media. At the end of each con-
centration-response curve, papaverine (10^{-4} \text{M}) was applied to attain the maximum relaxation, which was taken as 100%. In tests with antagonists, the maximum relaxation obtained with norepinephrine alone was set as 100%, and subsequent concentration-response curves in the presence of increasing concentrations of antagonists were expressed as a percentage of the maximum in the control curve. After two reproducible control curves had been obtained, an antagonist was pretreated for 30 min before responses to norepinephrine were examined. The log concentration-ratio of EC_{50} values (i.e., concentration producing half-maximum response) in the absence or presence of antagonist was calculated and plotted against the logarithm of antagonist concentration to obtain the pA_{2} values (7).

The endothelial cells of the arterial ring segments were mechanically removed by gentle rubbing of the intimal surface with a stainless rod having a diameter equivalent to the lumen of the artery. The presence or absence of the endothelial cells was determined morphologically by scanning and transmission electron microscopies and pharmacologically by testing the relaxation response to bradykinin and prostaglandin F2α. The amplitude of the relaxation in the artery precontracted with prostaglandin F2α (10^{-7}−10^{-6} \text{M}) was 50-60% of the high potassium (60 mM)-induced contraction. The amplitude of the contraction produced by prostaglandin F2α (10^{-7}−10^{-6} \text{M}) was 50-60% of the high potassium (60 mM)-induced contraction. The amplitude of the relaxation in the artery precontracted with prostaglandin F2α was larger than that under the resting tension.

RESULTS

Norepinephrine-induced relaxation

Figure 1 (A and B) show typical concentration-dependent relaxations to norepinephrine (10^{-8}−10^{-4} \text{M}) in the same pig basilar artery under the optimal resting tension and under the partially contracted condition, respectively. The amplitude of the contraction produced by prostaglandin F2α (10^{-7}−10^{-6} \text{M}) was 50-60% of the high potassium (60 mM)-induced contraction. The amplitude of the relaxation in the artery precontracted with prostaglandin F2α was larger than that under the resting tension.

The responsiveness to norepinephrine in the basilar arteries obtained from regions 1-4 (Fig. 1C) did not show any significant differences (data not shown). The artery from the 4th region was used in testing the relaxation response to norepinephrine, and all regions were used in a radioligand binding assay.

Removal of endothelium

Norepinephrine-induced relaxations were not significantly different in arteries with and without endothelium (data not shown). Therefore, the following experiments were done using the arteries with the endothelium.

Drugs used were as follows: [3H]-dihydroalprenolol (Dupont New England Nuclear, Specific activity; 107 Ci/mmol), (±)-norepinephrine (Tokyo Kasei), phentolamine mesylate (Ciba-Geigy), ICI 118,551 (erythro[±]-1-[7-methylindan-4-yloxy]-3-isopropyl-aminobutan-2-ol hydrochloride), (±)-propranolol hydrochloride (ICI), atenolol, butoxamine hydrochloride, bradykinin acetate salt, polyethylenimine (Sigma), prostaglandin F2α (Ono), and papaverine hydrochloride (Nacalai).

The results shown in the text, table and figures are expressed as mean values±S.E.M. Statistical analyses were made by Student’s t-test or Scheffe’s method after one-way analysis of variance. The significance was established when the probability level was equal to, or less than, 5%.
Effects of propranolol and phentolamine on norepinephrine-induced relaxation

Figure 1D shows the effect of a non-selective β-antagonist, propranolol, on the norepinephrine-induced relaxation in pig basilar arteries. Propranolol (10^{-7} and 10^{-6} M) inhibited the norepinephrine-induced relaxation concentration-dependently. However, 10^{-5} M propranolol reversed the relaxations to contractions. These contrac-
tions were blocked by a non-selective $\alpha$-antagonist, phentolamine ($10^{-5}$ M). Therefore, the following experiments were undertaken in the presence of phentolamine ($10^{-5}$ M) to exclude the participation of $\alpha$-adrenoceptors.

**Effects of selective $\beta$-antagonists**

Figure 2 shows the effects of propranolol, atenolol ($\beta_1$-antagonist), butoxamine ($\beta_2$-antagonist) and ICI 118,551 ($\beta_2$-antagonist) on the norepinephrine-induced relaxation. Figure 3 shows the Schild plots of these antagonists for the relaxation response to norepinephrine. These antagonists shifted the concentration-response curve for norepinephrine to right in parallel fashion in pig basilar artery. The slope values of the Schild plots for propranolol, atenolol, butoxamine and ICI 118,551 against norepinephrine were $0.82\pm0.21$, $1.18\pm0.31$, $0.92\pm0.28$ and $1.07\pm0.28$, respectively, which were not significantly different from unity. The calculated $pA_2$ values of each antagonist were $8.34\pm0.21$, $6.59\pm0.20$, $5.25\pm0.13$ and $6.60\pm0.18$, respectively.

**Binding of $[3H]$-DHA to the membrane fraction from pig basilar arteries**

Figure 4 shows a typical pattern obtained in the binding assay of $[3H]$-DHA to membrane fractions from pig basilar arteries in the absence (total binding) and presence (non-specific binding) of $100\ \mu$M propranolol. The specific binding, which was calculated as the difference between the total and non-specific bindings, appeared to be saturable. From 4 experiments, the equilibrium dissociation constant ($K_d$) value was determined to be $1.4\pm0.5$ nM and the binding capacity ($B_{max}$) was $48.7\pm5.6$ fmol/mg protein. The Scatchard plot of the specific binding gave a single line. The Hill coefficient of binding for the experiments was $0.99\pm0.02$, which was not significantly different from unity.

Figures 5 and 6 show competition curves for propranolol, atenolol, butoxamine and ICI 118,551 to specific $[3H]$-DHA binding and their Hofstee plots, respectively. The competition curve for propranolol showed a linear Hofstee plot, while the curves for atenolol, butoxamine and ICI 118,551 were biphasic. The pseudo-Hill coefficient calculated from the inhibition of $[3H]$-DHA binding by propranolol was $1.04\pm0.07$, which was not significantly different from unity, while the values obtained for atenolol, butoxamine and ICI 118,551 were $0.54\pm0.07$, $0.70\pm0.11$ and $0.71\pm0.15$, respectively, which were significantly less than unity.

Table 1 shows $K_i$ values of the high ($K_{hi}$) and low ($K_{li}$) affinity sites and the ratio of $\beta_1$- and $\beta_2$-adrenoceptor subtypes. The averaged concentration of $\beta_1$- and $\beta_2$-adrenoceptors in pig basilar arteries was $65\%$ and $35\%$, respectively.

Figure 7 shows the correlations with $pK_i$ values and $pA_2$.
Fig. 4. [\textsuperscript{3}H]-Dihydroalprenolol (DHA) binding to membrane fractions from pig basilar arteries (A) and Scatchard plot (B). Membrane fractions were incubated with increasing concentrations of [\textsuperscript{3}H]-DHA (0.25 – 5.0 nM) in the absence (total binding: ○) and presence (non-specific binding: △) of propranolol (10^{-4} M). Specific binding (●) was determined as the difference between non-specific and total bindings. The figure shows a typical saturation experiment.

Fig. 5. Inhibition of specific [\textsuperscript{3}H]-dihydroalprenolol (DHA) binding to membrane fractions from pig basilar arteries by propranolol, atenolol, butoxamine and ICI 118,551. The ordinate shows [\textsuperscript{3}H]-DHA binding expressed as a percentage of specific [\textsuperscript{3}H]-DHA binding. The values are the mean of 3 to 4 experiments done in duplicate.

Fig. 6. Hofstee plots for the inhibition of specific [\textsuperscript{3}H]-dihydroalprenolol (DHA) bound by propranolol, atenolol, butoxamine and ICI 118,551. The ordinate shows % inhibition of specific [\textsuperscript{3}H]-DHA binding by propranolol, atenolol, butoxamine and ICI 118,551 expressed as the average of 3 to 4 determinations. The abscissa shows % inhibition divided by the concentration of each antagonist. The values are the mean of 3 to 4 experiments done in duplicate.
values for $\beta_1$ and $\beta_2$-adrenoceptors, respectively. The pA₂ values for the four $\beta$-antagonists were significantly correlated with their pKᵢ values for $\beta_1$-adrenoceptors but not for $\beta_2$-adrenoceptors. The correlation coefficient for their relationship was 0.98.

**DISCUSSION**

The regional difference of the responsiveness to norepinephrine has been reported in bovine cerebral arteries (5). In the present study, however, the responsiveness to norepinephrine in the pig basilar arteries obtained from 4 regions was not significantly different from each other (data not shown). Steinberg et al. (13) have reported that cultured bovine aortic endothelial cells may contain $\beta$-adrenoceptors; however, in pig basilar artery, the removal of endothelial cells has no significant effect on the norepinephrine-induced relaxation in the present study. These results suggest that there was no regional difference in the distribution of $\beta$-adrenoceptor on the vascular smooth muscle cells of the pig basilar artery. A non-selective $\beta$-antagonist, propranolol ($10^{-7} - 10^{-6}$ M), inhibited the norepinephrine-induced relaxation concentration-dependently; and the pretreatment with $10^{-5}$ M propranolol converted the relaxation to contractions, which were blocked by a non-selective $\alpha$-antagonist, phentolamine ($10^{-5}$ M) (Fig. 1D). These results suggest that the relaxation induced by norepinephrine is predominantly mediated through the stimulation of $\beta$-adrenoceptors, and a few $\alpha$-adrenoceptors might modify the norepinephrine-induced relaxations.

Propranolol, atenolol ($\beta_1$-antagonist), butoxamine ($\beta_2$-antagonist) and ICI 118,551 ($\beta_2$-antagonist) competitively inhibited the norepinephrine-induced relaxation in pig basilar arteries (Fig. 2), and the slope values of Schild plots were not significantly different from unity (Fig. 3). Butoxamine and ICI 118,551 were typical selective $\beta_2$-antagonists; however, high concentrations of these antagonists are known to affect not only $\beta_2$-adrenoceptors but also affect $\beta_1$-adrenoceptors. The pA₂ values for butoxamine and ICI 118,551 are agreed well with pA₂ or pKᵢ values reported for $\beta_1$-adrenoceptors rather than $\beta_2$-adrenoceptors,

| Antagonist   | $K_i$               | $K_{H}$ (M) | $K_L$ (M) | $\beta_1$ (%) | $\beta_2$ (%) |
|-------------|---------------------|------------|-----------|---------------|---------------|
| Propranolol | $1.10 \pm 0.25 \times 10^{-7}$ | $2.00 \pm 1.65 \times 10^{-5}$ | $65 \pm 12$ | $35 \pm 12$   |
| Atenolol    | $6.66 \pm 1.40 \times 10^{-7}$ | $2.00 \pm 1.65 \times 10^{-5}$ | $65 \pm 4$  | $33 \pm 4$    |
| Butoxamine  | $1.47 \pm 0.56 \times 10^{-7}$ | $1.97 \pm 0.59 \times 10^{-6}$ | $67 \pm 4$  | $33 \pm 4$    |
| ICI 118,551 | $3.40 \pm 1.56 \times 10^{-6}$ | $1.26 \pm 0.35 \times 10^{-7}$ | $66 \pm 4$  | $34 \pm 6$    |

The values are expressed as the mean ± S.E.M. of 3 to 4 experiments done in duplicate. $K_{H}$: high affinity site. $K_L$: low affinity site.

![Fig. 7](image-url)
adrenoceptors in other tissues (9, 14–16). These results strongly suggest that $\beta_1$-adrenoceptors predominantly mediated the norepinephrine-induced relaxation in pig basilar arteries.

The radioligand binding assay was conducted to quantify the distribution of $\beta_1$- and $\beta_2$-adrenoceptors. The specific $[^3H]$-DHA binding to $\beta$-adrenoceptor in membrane fractions from pig basilar arteries was saturable ($B_{\text{max}} = 48.7 \text{ fmol/mg protein}$), reversible and of high affinity ($K_a = 1.4 \text{ nM}$) (Fig. 4). The Scatchard plot of the specific binding gave a single line (Fig. 4), and the Hill coefficient of $[^3H]$-DHA binding was not significantly different from unity. These results indicate that $[^3H]$-DHA binds to a single class of noncooperative sites. The competition curve for propranolol shows a linear Hofstee plot; however, the curves for atenolol, butoxamine and ICI 118,551 were biphasic (Fig. 6). The pseudo-Hill coefficient calculated from the inhibition of $[^3H]$-DHA binding by propranolol was not significantly different from unity, but the ones from atenolol, butoxamine and ICI 118,551 were significantly less than unity. The biphasic Hofstee plots are consistent with a model assuming the presence of two binding sites with different affinities. These results suggest that $\beta_1$- and $\beta_2$-adrenoceptors were present in pig basilar artery membranes. A $\beta_1$-$\beta_2$-adrenoceptor ratio was determined by analyzing the biphasic Hofstee plots with the computer program LIGAND (12). Computer analysis gave a $\beta_1$-$\beta_2$-adrenoceptor ratio of approximately 65:35 (Table 1). The $pK_I$ values for $\beta_1$-adrenoceptor, but not for $\beta_2$-adrenoceptor, were found to correlate significantly with the $pA_2$ values of propranolol, atenolol, butoxamine and ICI 118,551 in antagonizing the norepinephrine-induced relaxation of pig basilar artery (Fig. 7). These results suggest that the $pA_2$ values of $\beta_2$-antagonists, butoxamine and ICI 118,551, were ones for $\beta_2$-adrenoceptors but not for $\beta_2$-adrenoceptors.

In conclusion, the present study suggests that the relaxation response to norepinephrine is predominantly mediated through the stimulation of $\beta_1$-adrenoceptors on vascular smooth muscle cells in pig basilar artery. These results were similar to those obtained from pig coronary arteries (15, 16).

REFERENCES
1. Toda, N.: Alpha adrenergic receptor subtypes in human, monkey and dog cerebral arteries. J. Pharmacol. Exp. Ther. 226, 861–868 (1983)
2. Usui, H., Kurahashi, K., Shirahase, H., Fukui, K. and Fujiwara, M.: Endothelium-dependent vasconstriction in response to noradrenaline in the canine cerebral artery. Japan. J. Pharmacol. 44, 228–231 (1987)
3. Chang, J.Y., Hardebo, J.E. and Owman, Ch.: Differential vaso-motor action of noradrenaline, serotonin, and histamine in isolated basilar artery from rat and guinea-pig. Acta Physiol. Scand. 132, 91–102 (1988)
4. Duckles, S.P. and Bevan, J.A.: Pharmacological characterization of adrenergic receptors of a rabbit cerebral artery in vitro. J. Pharmacol. Exp. Ther. 197, 371–378 (1976)
5. Ayajiki, K. and Toda, N.: Isolated bovine cerebral arteries from rostral and caudal regions: distinct responses to adrenoceptor agonists. Eur. J. Pharmacol. 191, 417–425 (1990)
6. Lee, T.J.F., Kinkead, L.R. and Sarwinski, S.: Norepinephrine and acetylcholine transmitter mechanisms in large cerebral arteries of the pig. J. Cereb. Blood Flow Metab. 2, 439–450 (1982)
7. Shimokawa, H., Kim, P. and Vanhoutte, P.M.: Endothelium-dependent relaxation to aggregating platelets in isolated basilar arteries of control and hypercholesterolemic pigs. Circ. Res. 63, 604–612 (1988)
8. Nakane, T., Tsujimoto, G., Hashimoto, K. and Chiba, S.: Beta adrenoceptors in the canine large coronary arteries: beta-1 adrenoceptors predominate in vasodilation. J. Pharmacol. Exp. Ther. 245, 936–943 (1988)
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)
10. Bruns, R.F., Lawson-Wendling, K. and Pugsley, T.A.: A rapid filtration assay for soluble receptors using polyethyleneimine-treated filters. Anal. Biochem. 132, 74–81 (1983)
11. Munson, P.J. and Rodbard, D.: LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107, 220–239 (1980)
12. Steinberg, S.F., Jaffe, E.A. and Bliezkin, J.P.: Endothelial cells contain beta adrenoceptors. Naunyn Schmiedebergs Arch. Pharmacol. 325, 310–313 (1984)
13. Toda, N. and Okamura, T.: Beta adrenoceptor subtype in isolated human, monkey and dog epicardial coronary arteries. J. Pharmacol. Exp. Ther. 253, 518–524 (1990)
14. Yamada, S., Kashiwabara, T., Yamazawa, T., Harada, Y. and Nakayama, K.: Demonstration of $\beta_1$-adrenoceptor mediating relaxation of porcine coronary artery by radioligand binding and pharmacological methods. Life Sci. 43, 1999–2006 (1988)
15. Schwartz, J. and Velly, J.: The $\beta_1$-adrenoceptor of pig coronary arteries: determination of $\beta_1$ and $\beta_2$ subtypes by radioligand binding. Br. J. Pharmacol. 79, 409–414 (1983)