Characterization of β-Adrenoceptors in Pig Basilar Artery from Functional and Radioligand Binding Studies

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ABSTRACT—β-Adrenoceptors in pig basilar arteries were investigated by measuring the relaxation responses to norepinephrine and by a radioligand binding assay with [^{3}H]-dihydroalprenolol (DHA). Norepinephrine induced concentration-dependent relaxations. The relaxation responses were independent of the presence of endothelial cells, and they were competitively antagonized by (±)-propranolol, atenolol, butoxamine and ICI 118,551. Specific [^{3}H]-DHA binding to β-adrenoceptors was saturable, reversible and high affinity (K_d = 1.4 nM), with a B_{max} of 48.7 fmol/mg protein. Computer analysis of inhibition of [^{3}H]-DHA binding by atenolol, butoxamine and ICI 118,551 gave a β_1:β_2-adrenoceptor ratio of approximately 65:35. The pA_2 values of these antagonists were significantly correlated with the K_i values for β_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 β_1-adrenoceptors on vascular smooth muscle cells in a pig basilar artery.

Keywords: Basilar artery (pig), Relaxation, β_1-Adrenoceptor, Norepinephrine, [^{3}H]-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 β-adrenoceptors. However, the β-adrenoceptor subtype responsible for the relaxation of pig basilar arteries has not been determined.

The present study was undertaken to clarify the distribution of β-adrenoceptor subtypes and the functional roles of these receptors in pig basilar artery, by measuring the effects of β_1- and β_2-antagonists on both the norepinephrine-induced relaxation response and the specific [^{3}H]-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_2PO_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℃ (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-

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centration-response curve, papaverine (10^{-4} 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 ($10^{-8}-10^{-6}$ M), which was abolished by the endothelium denudation (8).

Isolated pig basilar arteries for a radioligand binding assay were cut longitudinally and then rinsed with the physiological salt solution. The rinsed basilar arteries were minced with scissors and then homogenized in 8 volumes of 50 mM Tris-HCl buffer (pH 7.4) by using a Polytron homogenizer at setting 8 for 8 periods of 15 sec with 45 sec intervals in an ice bath. The membrane fraction of basilar arteries was prepared according to the method by Nakane et al. (9). Briefly, the homogenate was centrifuged at $470\times g$ for 15 min. Then the supernatant was centrifuged at $100{,}000\times g$ for 30 min. The pellet was resuspended in 50 mM Tris-HCl phosphate buffer solution, and the suspension was used for the binding assay as a crude membrane fraction. These procedures were all performed at a temperature 4℃. The protein concentration of the final suspension was measured by the method of Lowry et al. (10) with bovine serum albumin as a standard.

Aliquots (approximately 300 μg of protein) of the membrane fraction were incubated with 0.25–5.0 nM of [^{3}H]-DHA in the presence or absence of 100 μM propranolol. After a 40-min incubation at 25℃, membrane-bound ligand was separated from unbound ligand by rapid filtration through a glass fiber filter (GF/C, Whatman), which had been presoaked in 0.3% polyethylenimine solution to eliminate nonspecific binding to the filter (11). The filters were immediately washed 3 times with 5 ml of ice-cold Tris-buffer. Tissue-bound radioactivity was extracted from the filters in scintillation fluid (0.5 liter of toluene and 0.5 liter of ethyl cellosolve, 7 g of PPO and 1 g of POPOP), and radioactivity was counted by a liquid scintillation counter (LSC-3050, Aloka Co.). Specific [^{3}H]-DHA binding was defined as the difference between the binding in the absence and presence of 100 μM propranolol. The equilibrium dissociation constant (K_d) value and the maximum binding capacity (B_{max}) were calculated from Scatchard plots. In the competition experiment, aliquots of the membrane fraction were incubated in the presence of various concentrations of a β-antagonist together with 0.8 nM [^{3}H]-DHA. Competition curves were analyzed by the computer program LIGAND (12).

Drugs used were as follows: [^{3}H]-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 $F_{2\alpha}$ (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 Scheffé's method after one-way analysis of variance. The significance was established when the probability level was equal to, or less than, 5%.

RESULTS

Norepinephrine-induced relaxation

Figure 1 (A and B) show typical concentration-dependent relaxations to norepinephrine ($10^{-8}-10^{-4}$ 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 $F_{2\alpha}$ ($10^{-7}-10^{-6}$ M) was 50–60% of the high potassium (60 mM)-induced contraction. The amplitude of the relaxation in the artery precontracted with prostaglandin $F_{2\alpha}$ 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.

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-

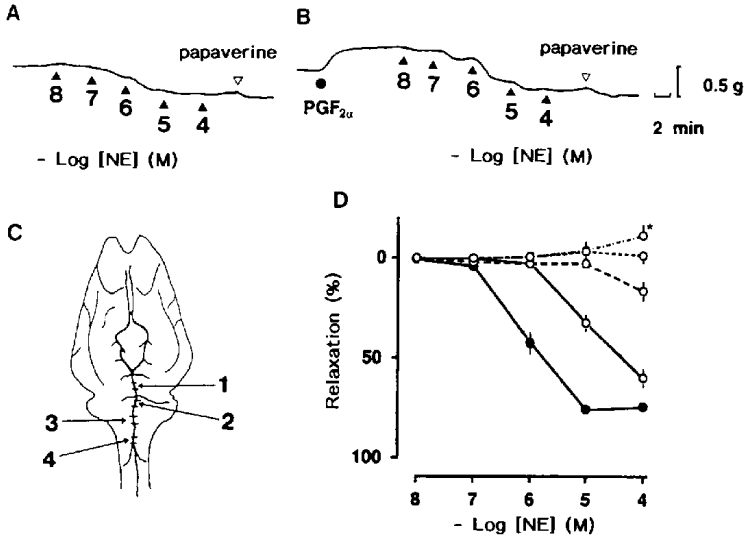

Fig. 1. Typical relaxation responses to norepinephrine in basilar arteries (4 in C) obtained from the same pig under the optimal resting tension (A) and under the precontracted condition with $PGF_{2\alpha}$ (B). Norepinephrine was added to the bath at the indicated points; figures indicate the cumulative concentration ($-\log$ M). The responses in the arteries obtained from regions 1–4 (C) did not show any significant differences. Effects of propranolol and phentolamine on norepinephrine-induced relaxation in pig basilar arteries (4 in C) precontracted with $PGF_{2\alpha}$ (10^{-7}–10^{-6} M) (D). Relaxations induced by 10^{-4} M papaverine was taken as 100%; the mean absolute value in arteries was 432±53 mg. The values are expressed as the mean±S.E.M. of 8 different animals. Control (—●—); propranolol (—○—: 10^{-7} M, – –○– –: 10^{-6} M, ···○···: 10^{-5} M); 10^{-5} M propranolol plus 10^{-5} M phentolamine (---○---). *: 10^{-4} M norepinephrine caused significant contraction ($P<0.05$) in the presence of 10^{-5} M propranolol; the mean absolute value was 49±24 mg. NE: norepinephrine, $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$.

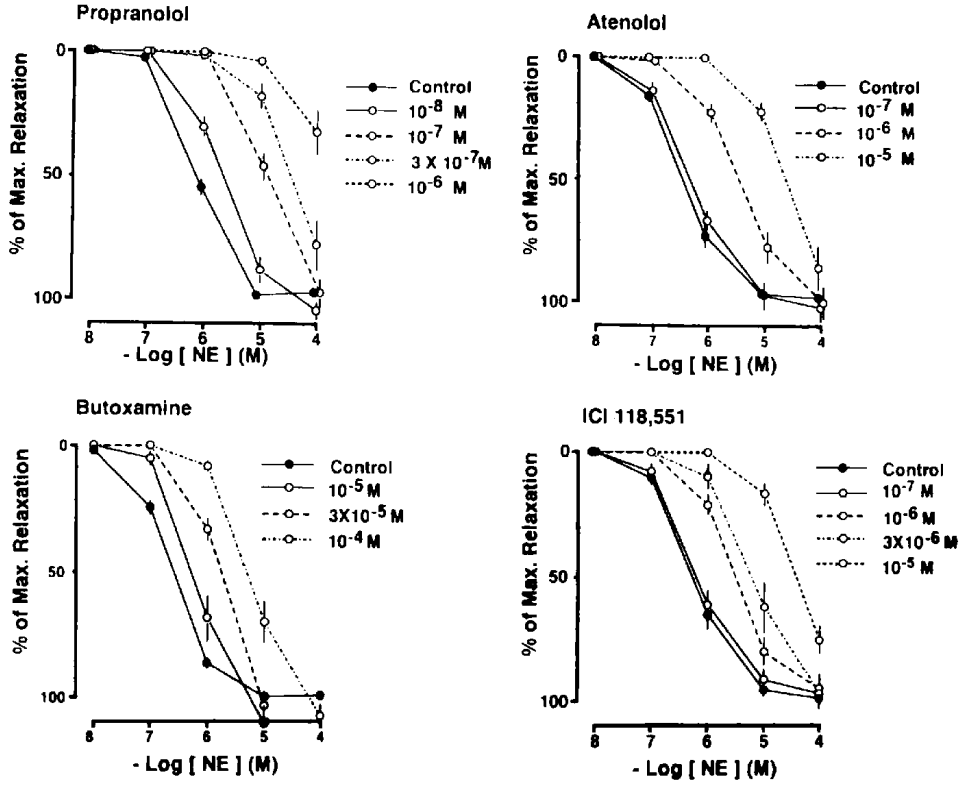

Fig. 2. Effects of propranolol, atenolol, butoxamine and ICI 118,551 on norepinephrine-induced relaxation in pig basilar arteries. The maximum relaxation induced by norepinephrine was taken as 100%. The values are expressed as the mean±S.E.M. of 8 different animals. NE: norepinephrine.

tions were blocked by a non-selective α-antagonist, phentolamine (10^{-5} M). Therefore, the following experiments were undertaken in the presence of phentolamine (10^{-5} M) to exclude the participation of α-adrenoceptors.

Effects of selective β-antagonists

Figure 2 shows the effects of propranolol, atenolol (β_1-antagonist), butoxamine (β_2-antagonist) and ICI 118,551 (β_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 ± 0.21, 1.18 ± 0.31, 0.92 ± 0.28 and 1.07 ± 0.28, respectively, which were not significantly different from unity. The calculated pA_2 values of each antagonist were 8.34 ± 0.21, 6.59 ± 0.20, 5.25 ± 0.13 and 6.60 ± 0.18, respectively.

Binding of [^{3}H]-DHA to the membrane fraction from pig basilar arteries

Figure 4 shows a typical pattern obtained in the binding assay of [^{3}H]-DHA to membrane fractions from pig basilar arteries in the absence (total binding) and presence (non-specific binding) of 100 μM 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 ± 0.5 nM and the binding capacity (B_{max}) was 48.7 ± 5.6 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 ± 0.02, which was not significantly different from unity.

Figures 5 and 6 show competition curves for propranolol, atenolol, butoxamine and ICI 118,551 to specific [^{3}H]-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 [^{3}H]-DHA binding by propranolol was 1.04 ± 0.07, which was not significantly different from unity, while the values obtained for atenolol, butoxamine and ICI 118,551 were 0.54 ± 0.07, 0.70 ± 0.11 and 0.71 ± 0.15, respectively, which were significantly less than unity.

Table 1 shows K_i values of the high (K_H) and low (K_L) affinity sites and the ratio of β_1- and β_2-adrenoceptor subtypes. The averaged concentration of β_1- and β_2-adrenoceptors in pig basilar arteries was 65% and 35%, respectively.

Figure 7 shows the correlations with pK_i values and pA_2

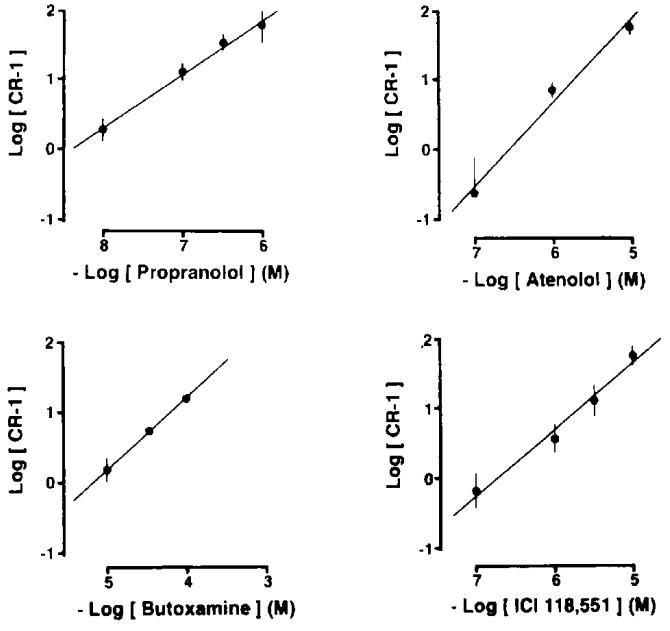

Fig. 3. Schild plots of propranolol, atenolol, butoxamine and ICI 118,551 for relaxation responses to norepinephrine in basilar arteries. The values are expressed as the mean $\pm$ S.E.M. of 8 different animals. The slopes of the Schild plot for these antagonists were not significantly different from unity. CR: An equieffective concentration-ratio of norepinephrine, i.e., concentration of agonist producing 50% maximal response (EC_{50}) in the presence of antagonist/EC_{50} in the absence of antagonist.

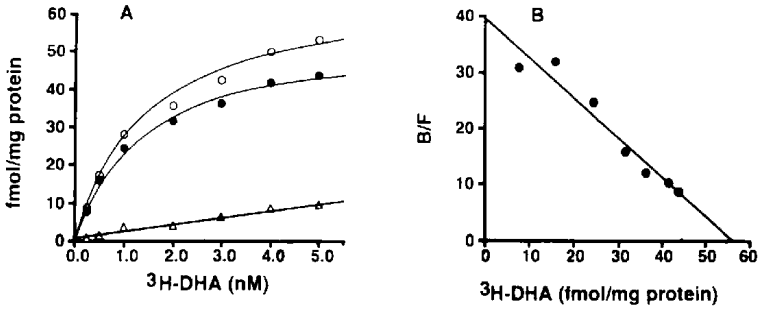

Fig. 4. [^{3}H]-Dihydroalprenolol (DHA) binding to membrane fractions from pig basilar arteries (A) and Scatchard plot (B). Membrane fractions were incubated with increasing concentrations of [^{3}H]-DHA (0.25 – 5.0 nM) in the absence (total binding: ○) and presence (non-specific binding: △) of propranolol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

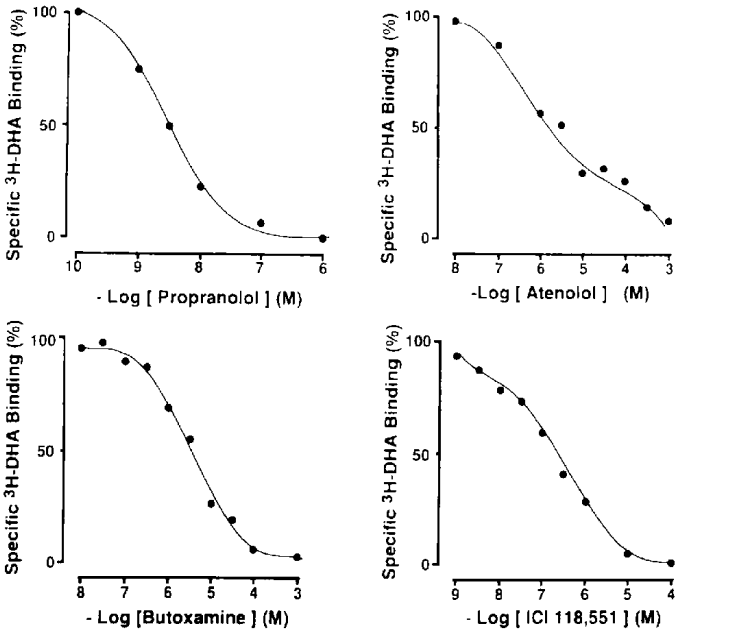

Fig. 5. Inhibition of specific [^{3}H]-dihydroalprenolol (DHA) binding to membrane fractions from pig basilar arteries by propranolol, atenolol, butoxamine and ICI 118,551. The ordinate shows [^{3}H]-DHA binding expressed as a percentage of specific [^{3}H]-DHA binding. The values are the mean of 3 to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

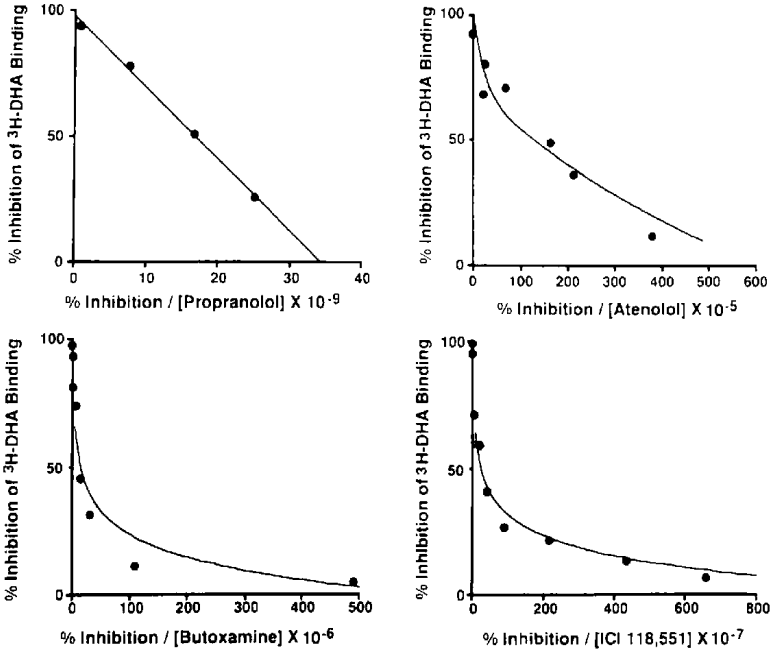

Fig. 6. Hofstee plots for the inhibition of specific [^{3}H]-dihydroalprenolol (DHA) bound by propranolol, atenolol, butoxamine and ICI 118,551. The ordinate shows % inhibition of specific [^{3}H]-DHA binding by propranolol, atenolol, butoxamine and ICI 118,551 expressed as the average of 3 to 4 determinations. The abscissa shows % inhibition divided by the concentration of each antagonist. The values are the mean of 3 to 4 experiments done in duplicate.

Table 1. Effects of β-antagonists on specific [^{3}H]-dihydroalprenolol binding in pig basilar arteries and relative percentages of β_1- and β_2-adrenoceptor subtypes

Antagonist	K_i		β_1 (%)	β_2 (%)
	K_H (M)	K_L (M)		
Propranolol	$1.10 \pm 0.25 \times 10^{-7}$			
Atenolol	$6.66 \pm 1.40 \times 10^{-7}$	$2.00 \pm 1.65 \times 10^{-5}$	65 ± 12	35 ± 12
Butoxamine	$1.47 \pm 0.56 \times 10^{-7}$	$1.97 \pm 0.59 \times 10^{-6}$	67 ± 4	33 ± 4
ICI 118,551	$3.40 \pm 1.56 \times 10^{-9}$	$1.26 \pm 0.35 \times 10^{-7}$	66 ± 6	34 ± 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.

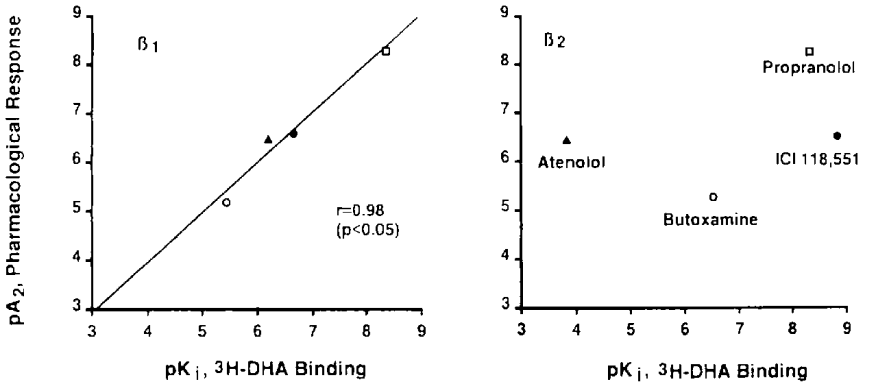

Fig. 7. Comparison of pK_i values of β-antagonists for β_1- and β_2-adrenoceptors with pA_2 values for the inhibition of relaxation response to norepinephrine in basilar arteries isolated from pigs. The values are expressed as the mean value. The pA_2 values for the four β-antagonists significantly ($P < 0.05$) correlated with the pK_i values for β_1-adrenoceptors; The correlation coefficient (r) for this relationship was 0.98.

values for β_1- and β_2-adrenoceptors, respectively. The pA_2 values for the four β-antagonists were significantly correlated with their pK_i values for β_1-adrenoceptors but not for β_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 β-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 β-adrenoceptor on the vascular smooth muscle cells of the pig basilar artery.

A non-selective β-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 α-antagonist, phentolamine (10^{-5} M) (Fig. 1D). These results suggest that the relaxation induced by norepinephrine is predominantly mediated through the stimulation of β-adrenoceptors, and a few α-adrenoceptors might modify the norepinephrine-induced relaxations.

Propranolol, atenolol (β_1-antagonist), butoxamine (β_2-antagonist) and ICI 118,551 (β_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 β_2-antagonists; however, high concentrations of these antagonists are known to affect not only β_2-adrenoceptors but also affect β_1-adrenoceptors. The pA_2 values for butoxamine and ICI 118,551 are agreed well with pA_2 or pK_i values reported for β_1-adrenoceptors rather than β_2-

adrenoceptors in other tissues (9, 14–16). These results strongly suggest that β_1-adrenoceptors predominantly mediated the norepinephrine-induced relaxation in pig basilar arteries.

The radioligand binding assay was conducted to quantify the distribution of β_1- and β_2-adrenoceptors. The specific [^{3}H]-DHA binding to β-adrenoceptor in membrane fractions from pig basilar arteries was saturable (B_{max}=48.7 fmol/mg protein), reversible and of high affinity (K_d=1.4 nM) (Fig. 4). The Scatchard plot of the specific binding gave a single line (Fig. 4), and the Hill coefficient of [^{3}H]-DHA binding was not significantly different from unity. These results indicate that [^{3}H]-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 [^{3}H]-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 β_1- and β_2-adrenoceptors were present in pig basilar artery membranes. A β_1:β_2-adrenoceptor ratio was determined by analyzing the biphasic Hofstee plots with the computer program LIGAND (12). Computer analysis gave a β_1:β_2-adrenoceptor ratio of approximately 65:35 (Table 1). The pk_i values for β_1-adrenoceptor, but not for β_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 β_2-antagonists, butoxamine and ICI 118,551, were ones for β_1-adrenoceptors but not for β_2-adrenoceptors.

In conclusion, the present study suggests that the relaxation response to norepinephrine is predominantly mediated through the stimulation of β_1-adrenoceptors on vascular smooth muscle cells in pig basilar artery. These results were similar to those obtained from pig coronary arteries (15, 16).

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