Pharmacological Characterization of Adrenergic Alpha and Beta Receptors Mediating the Vasomotor Responses of Cerebral Arteries In Vitro

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ABSTRACT

The adrenergic receptors in the isolated feline middle cerebral artery were characterized pharmacologically using a sensitive system for recording circular contractions in vitro. Epinephrine, norepinephrine, isoproterenol, and phenylephrine contracted the vessel in the mentioned order of potency. Together with the inhibitory patterns obtained with graded doses of piperoxan (reversible competitive inhibition) and dibenamine or phenoxybenzamine (irreversible competitive inhibition), these results showed that the contraction was mediated by alpha receptors. With piperoxan and norepinephrine, the mean value for $\mathrm{pA}_2$ was 7.06 and for $K_H > 10^{-7} \text{M}$. The mean value for $K_A$ calculated for norepinephrine before and after partial irreversible blockade of the alpha receptors with phenoxybenzamine was $1.73 \times 10^{-7} \text{M}$. The norepinephrine response was not directly proportional to the amount of receptors occupied; $\mathrm{ED}_{50}$ was reached when only about 11% of the receptors were occupied and the $\mathrm{E}_{hm}$ response was obtained when 75% of the receptors were occupied. Dilation was achieved only after an active tonic contraction had been induced (with 5-hydroxytryptamine) in the vessels, and the order of potency was isoproterenol > norepinephrine = epinephrine > terbutaline. Inhibition with INPEA and propranolol was competitive, as confirmed by Arunlakshana-Schild plots, showing that the dilatory response was a beta-receptor effect. The values for $\mathrm{pA}_2$ (8.78 and 9.17) and $K_B$ ($2.31 \times 10^{-9} \text{M}$ and $1.77 \times 10^{-9} \text{M}$) for propranolol were indistinguishable in tests with terbutaline and isoproterenol, respectively. Comparison of the relative potencies of norepinephrine and epinephrine as well as isoproterenol and terbutaline suggested that the receptors were of the beta, type.

KEY WORDS: adrenoceptors, INPEA, terbutaline, propranolol, dissociation constants

It is now well established that the pial vascular system and the intracerebral vessels receive an ample supply of sympathetic adrenergic nerves (1, 2). In comparison with other vascular beds, this supply is very pronounced in terms of the number of axon terminals relative to the local amount of smooth musculature in a given portion of the vessel wall. Chemical determinations on pial vascular tissue (3) have confirmed the presence of norepinephrine in amounts corresponding to those reported for well-innervated peripheral arterial vessels (4). Ultrastructurally, the neuromuscular complex fulfills accepted morphological criteria for true sympathetic vascular innervation both at the level of the extracerebral (5) and the intracerebral (6) arterial system. Functionally, it has been shown in experiments with tyramine (7) and electric field stimulation (8) that the stored norepinephrine transmitter is present in the perivascular nerves in amounts sufficient to elicit a local vasomotor response on release. For a number of years, attempts have been made to demonstrate a sympathetic neurogenic influence on cerebral blood flow through investigations involving nerve stimulation, denervation, and drug administration (1), but the results have often been inconsistent. A detailed knowledge of the adrenergic receptor mechanisms mediating the vasomotor responses of the brain vessels is a prerequisite for a proper understanding of the physiological role of sympathetic nerves and circulating catecholamines in the regulation of cerebral hemodynamics. Although such information is available for several peripheral vascular beds (9), little is known about cerebrovascular adrenoceptors, perhaps partly because early investigations failed to obtain catecholamine-induced vasomotor responses in isolated cerebral resistance vessels (10). However, using a sensitive in vitro system (11), Nielsen and Owman (12) have been able to record strong contractions induced by
several amines tested on the feline middle cerebral artery. Histochemical studies have shown that the extracerebral arteries are also innervated by cholinergic nerves which run in close association with the adrenergic fibers (13), and there is experimental evidence that the arteries possess not only adrenergic but also cholinergic, serotoninergic, and histaminergic receptors (14).

In the present study, the adrenergic alpha and beta receptors were characterized in that part of the cerebral vascular bed represented by the pial vessels. The isolated middle cerebral artery of the cat was chosen as the model for an in vitro analysis of changes in the mechanical dose-response patterns occurring in the presence of various sympathomimetic compounds before and after graded blockade with various specific antagonists under standardized conditions.

**Methods**

**ANIMALS**

About 100 cats of either sex (2.0-5.2 kg) were anesthetized with sodium pentobarbital (30 mg/kg, ip) and killed by exsanguination and decapitation. The skull was opened, and the brain was removed, placed in a Petri dish, and soaked in a Krebs-Ringer's buffer solution at room temperature.

To avoid any interference caused by uptake of administered agonists into the perivascular adrenergic nerve plexus, the arterial preparations used in the quantitative tests were subjected to postganglionic sympathetic denervation by removal of the superior cervical ganglia 1 week prior to the experiment (15). The outcome of the sympathectomy was checked by fluorescence microscopy of formaldehyde-treated stretch-preparations (15) from that part of the middle cerebral artery not mounted in the organ bath.

**PIAL ARTERY PREPARATION**

Immediately after removal of the brain, 5 mm of the proximal part of each middle cerebral artery was dissected out and mounted in a 50-ml mantled organ bath with two separate systems of metal holders for recording circular contractions and Endevco model 8107-2 force-displacement transducers (11). The isotonic contractions were amplified and recorded on a Grass model 7B polygraph. Mounting of the artery in the organ bath was complete within 15 minutes after removal of the brain.

The bath contained a buffer solution of the following composition: NaCl 118, KCl 4.5, CaCl₂ 2H₂O 2.5, MgSO₄⋅7H₂O 1.0, NaHCO₃ 25; KH₂PO₄ 1.0, and glucose 6.0. The bath and the stock buffer solution were maintained at 37.5 ± 0.5°C; temperature was continuously checked by a needle thermocouple in the bath. A 95% O₂-5% CO₂ mixture was used for continuous aeration, and the flow of the gas was set to give a pH of 7.387 ± 0.002 (SE). The pH was determined in 50-μlinter samples removed from the organ bath immediately before and after injection of each dose of test compound, using a Radiometer pH meter 27 with a type E5021 electrode unit. Additional details about the experimental setup have been published previously (11).

Shortly after two arterial preparations had been mounted in the organ bath, each was subjected to a load of 400 dynes and allowed to stabilize; tension decreased approximately 50 dynes. The test drugs were administered after 2 hours of accommodation time. The basis for these experimental conditions has been reported elsewhere (11).

The structure and the dimensions of the pial artery preparations used in these tests were also studied using brains from an additional few cats. The brain was removed, and a 3% solution of gelatin (37°C) was injected into the middle cerebral artery via a small cannula in the posterior communicating artery. The brain was placed in a refrigerator at 0°C for 5 minutes to allow the gelatin to harden. In this way, collapsing of the artery was avoided. The artery and a piece of underlying brain were frozen to the chuck of a cryostat, sectioned transversely at -20°C into 20 μm thicknesses, and stained with hematoxyline and eosin.

**DRUGS**

L-Phenylephrine hydrochloride (Schwarz-Mann), L-arterenol hydrochloride or bitartrate monohydrate (Sigma), L-epinephrine bitartrate (Sigma or Calbiochem), L-isoproterenol hydrochloride (Sigma), terbutaline sulfate (Bricanyl, gift from Draco Ltd., Sweden), phenoxybenzamine hydrochloride (Dibenzyline, Smith-Kline and French), dibenamine hydrochloride (gift from Draco Ltd., Sweden), piperoxan hydrochloride (Rhône-Poulenc), DL-INPEA (N-isopropyl-p-nitrophenyl-ethanolamine, Selvi), propranolol chloride (Inderal, Scanmeda), cocaine hydrochloride (ACO, Sweden), normetanephrine hydrochloride (Sigma), acetycholine chloride (Calbiochem), carbachyolne chloride (Al drich), 5-hydroxytryptamine creatinine sulfate (Sigma), and angiotensin (Hypertensin N, Ciba) were used. These substances were dissolved in 0.9% saline. The catecholamine solutions contained 0.2 mg ascorbic acid/ml to minimize oxidation of the amine. All concentrations throughout this paper are given as the salt and expressed as the final molar concentration in the bath.

**METHODOLOGICAL CONSIDERATIONS**

The differentiation of adrenergic receptors in isolated tissues requires certain experimental precautions if quantitative measurements are to be properly interpreted. These experimental conditions have been discussed in detail by Furchgott (16, 17). For example, if the system can be expected to have both alpha and beta adrenoceptors and attempts are made to characterize one receptor type, the other receptor type must be blocked before agonists with actions on both receptor types are tested. Thus, in the present investigation the alpha receptors were blocked irreversibly with phenoxybenzamine or dibenamine, and, since no irreversible antagonist is available for beta receptors, propranolol was used when the alpha receptors were under study. Furthermore, since amines are actively removed from the region of the receptor by the adrenergic nerve terminals, the concentration near the receptor can be expected to become lower than that in the bath unless this "Uptake," process (18) is blocked. A separate study has shown that the isolated feline middle cerebral artery becomes three times more sensitive (comparison of ED₅₀ values) to norepinephrine in the presence of 10⁻⁴ M cocaine or 1 week after sympathectomy (19). Although the
affinity for the neuronal uptake site is relatively low for amines such as phenylephrine and isoproterenol (18). Attempts were made to standardize the present experimental conditions, when required, by using sympathetic denervation or constant exposure of a nonadenervated vessel to cocaine. At higher amine concentrations, when uptake is becoming saturated, it is still possible to demonstrate active removal of amines from the tissue (18): this process seems to involve transport of the amine into cells other than nerves. This nonneuronal process has been designated “Uptake,” (18), and in the present experiments it was inhibited by normetanephrine.

The preparation used in the present study—a segment from the middle cerebral artery 300–400µ in diameter—has previously been tested to determine the amount of initial tension (11) necessary to achieve an optimal contractile response to a given dose of agonist (norepinephrine). The tangential tension in the vessel wall in vivo is considerably lower than that exerted in vitro, showing that the vessel is not overstretched in our test system. Changes in sensitivity which occur during the course of application of various agonists to this preparation have also been studied previously (11), and a period of 2–4 hours long when sensitivity (measured as ED₅₀ values) and contractile force (in terms of E₅₀ values) are constant has been defined. The concentration of agonist in the external solution seems to be in diffusion equilibrium with that in the region of the receptors; pilot experiments have shown a close agreement between the dose-response curves obtained by cumulative application (20) and those obtained by fractionated application of the agonist (dose-response curves were measured from individual contractions and the preparation was rinsed frequently after each response had been recorded).

DEFINITIONS AND TREATMENT OF DATA

According to the occupation theory of drug-receptor interaction, the formation of the receptor-agonist complex, RA, is governed by the law of mass action. The response of a test preparation to an agonist under steady-state conditions is considered to be a function of the concentration of the receptor-agonist complex. [RA], times an efficacy term for that specific receptor-agonist complex. For equal responses before and after inactivation of a fraction of receptors by an irreversible type of blocking agent the following equation applies:

\[
\frac{1}{[A]_1} = \frac{1-q}{q} \times \frac{1}{K_A} + \frac{1}{[A']_1} \tag{1}
\]

A plot of 1/\([A]_1\) against 1/[A'] should therefore give a straight line from which K_A can be calculated from the relation

\[
K_A = \frac{\text{slope}}{\text{intercept for } 1/[A]_1} \tag{2}
\]

assuming that the same concentration of receptor-agonist complex always results in the same response.

At equilibrium, it is possible to express the receptor occupancy, which is the relation between the concentration of the receptor-agonist complex and the total amount of receptors, in the following way:

\[
\frac{[RA]}{[R_T]} = \frac{[A]}{[A] + K_A} \tag{3}
\]

To determine the value for the dissociation constant of the receptor-agonist complex at equilibrium during competitive inhibition, the following equation has been used:

\[
K_R = \frac{[B]}{[A']_1/[A]_1 - 1} \tag{4}
\]

The preceding symbols and equations were compiled from material by Furchgott (16, 17, 21, 22), Vaud (23), Johansson et al. (24), and Wenke (25); these references also provide additional details about the theoretical background for this type of investigation.

The dose-response relation in the various experiments was illustrated in terms of relative vasomotor response. In cases in which the antagonism of the response involved a decrease in the maximum and the slope of the actual log dose-response curves, the E₅₀ of the curve before addition of the antagonist was set as 100% and the subsequent curves obtained after blockade were related to this value. When antagonism only involved a parallel shift in the actual log dose-response curves, the E₅₀ of each curve from the experiment was set as 100%.

Calculations for the regression analysis and of differences between mean values (Student’s t-test) were performed using a Hewlett-Packard desk computer.

Results

STRUCTURE AND DIMENSIONS OF THE ARTERIAL PREPARATION

Measurements with a scale fitted in a light microscope showed that the proximal portion of the feline middle cerebral arteries used in the pharmacological tests had an outer diameter of 300–400µ and a wall thickness of 50µ. Smooth muscle oc-
cupied 40 μ of the wall thickness; usually five layers of muscle cells ran in an essentially circular manner around the wall. The artery was also equipped with a thin internal elastic membrane, and a 10 μ thick adventitia (in which the nerves ran) surrounded the musculature. The density of the adrenergic network was estimated by counting the number of meshes formed by the nerve terminals; there were 40-50 meshes/cross section of vessel.

Assuming blood pressure to be 80 mm Hg in the test artery, the transmural pressure in vivo would be 1,100 dynes/mm². According to the law of Laplace, this value corresponds to an in vivo tangential tension of 3,300 dynes/mm² in a vessel 350 μ in diameter. Considering the passive load applied to the vessel (400 dynes) and the way in which the vessel was mounted, the tension in the wall in vitro was 800 dynes/mm².

EXPERIMENTS ON RESTING ARTERIES

General Properties.—The vessels showed no spontaneous contractions either with or without sympathetic denervation. Under resting conditions, i.e., when the artery had achieved a steady level of tension after the 400-dyne load had been applied, epinephrine, norepinephrine, isoproterenol, and phenylephrine all produced a contractile response. After administration of each agonist dose to the organ bath, the contraction fairly rapidly reached a plateau, which was maintained long enough to allow cumulative application of increasing doses (see Fig. 1). Pilot experiments had established that the dose-response curves obtained after fractionated application of the drugs closely agreed with those resulting from cumulative application. After rinsing, the arteries returned to their initial level of tension. Terbutaline (tested up to a final bath concentration of 10⁻⁶ M) was usually without effect on the resting vessel; in a few instances it produced a slight relaxation of 50-100 dynes that started to appear when a concentration of approximately 10⁻⁴ M had been reached in the bath during the cumulative application. A very slight dilatory response (about 25 dynes) was also noted at times in the presence of isoproterenol in doses between 10⁻⁷ and 5 × 10⁻⁶ M (see Fig. 7C). Quantitative data were obtained from tests with sympathectomized vessels in the presence of 10⁻⁷ M propranolol (added to the stock buffer solution). The mean values for the maximum contractions (Eₐₘ) induced by epinephrine, norepinephrine, isoproterenol, and phenylephrine appear in Table 1.

Relative Potency.—To evaluate the relative potency of the amines with regard to their contractile effects, full log dose-response curves were constructed and the mean concentrations required for half-maximum contraction (ED₅₀) were compared. The ED₅₀ for norepinephrine was set as unity. The arteries were sympathetically denervated and the beta receptors were blocked by continuous exposure to 10⁻⁷ M propranolol. The relative potency was epinephrine > norepinephrine > isoproterenol > phenylephrine. The results are summarized in Table 1.

Partial Agonism.—The order and the degree of relative potency found for the amines mediating contraction in the feline middle cerebral artery are in accord with those reported for alpha responses in other tissues (17) with the exception that the ratio of phenylephrine potency to norepinephrine potency is usually about ten times higher than that presently obtained. Together with the finding of a lower order of potency for phenylephrine than for isoproterenol and the comparatively low Eₐₘ value for the phenylephrine-induced contractile effect, the low ratio probably indicates that phenylephrine is a partial agonist. This supposition was confirmed by further experiments in which the relative potencies of phenylephrine and norepinephrine were determined according to the “brack-

<table>
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<tr>
<th>Compound</th>
<th>n</th>
<th>Eₐₘ (dynes)</th>
<th>ED₅₀ (M)</th>
<th>Relative potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td>19</td>
<td>252 ± 48</td>
<td>(1.24 ± 1.86) · 10⁻⁴</td>
<td>1.75</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>22</td>
<td>233 ± 48</td>
<td>(2.17 ± 0.55) · 10⁻⁴</td>
<td>1</td>
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<tr>
<td>Isoproterenol</td>
<td>14</td>
<td>255 ± 66</td>
<td>(7.33 ± 1.36) · 10⁻⁴</td>
<td>0.030</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>23</td>
<td>161 ± 49</td>
<td>(9.78 ± 4.13) · 10⁻⁴</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Results are means ± se. The vessels were sympathetically denervated by bilateral removal of the superior cervical ganglion 1 week prior to testing and their beta receptors were blocked by continuous exposure to 10⁻⁷ M propranolol. Relative potency was based on a comparison of ED₅₀ values, assuming the value for norepinephrine to be unity. n = number of experiments. Eₐₘ = maximum contraction, and ED₅₀ = dose producing a half-maximum contraction.
etition procedure” (16). For these experiments, the vessels had previously been denervated by sympathectomy, and the buffer solution in the organ bath contained 10^{-6} M propranolol to block the beta receptors. As shown in Figure 1A, a cumulative dose-response curve was first determined with phenylephrine and, when maximum contraction had been reached, the preparation was washed and allowed to start relaxing. During relaxation, norepinephrine was added in a dose equal to the dose of phenylephrine that had produced the maximum contraction. This addition resulted in a contractile response that was markedly higher than the maximum response to phenylephrine. After several rinsings, the vessel returned to its original level of tension and was allowed to rest for 45 minutes. The procedure was then repeated but in the order norepinephrine, rinsing, phenylephrine. Phenylephrine was only able to produce a faint response compared with the contraction induced by an equal dose of norepinephrine; in fact the response was so faint that, during the relaxation phase following rinsing, phenylephrine did not cause any increased force of contraction but only a cessation of further relaxation (Fig. 1B).

Reversible Competitive Antagonists.—If the contractile response to the amines is mediated by alpha receptors, a reversible competitive antagonist should decrease the sensitivity of the test system to the various agonists without decreasing the maximum or the slope of the log dose-response curve. To test this supposition, piperoxan, which is a piperidine derivative of 1,4-benzodioxan (26), was used as the competitive antagonist. Piperoxan contracted the pial arteries when it was first applied (Fig. 2), but the tone of the vessel returned (in the presence of piperoxan) to the basal level within 10 minutes, i.e., before tests with the agonist were carried out in the subsequent experiments.

To allow for the quantitative estimations, interference from the beta receptors was avoided by adding propranolol (10^{-6} M) to the stock buffer solution. Any neuronal uptake of amine (Uptake_{1}) was excluded by sympathectomy or by the continuous presence of 10^{-4} M cocaine, and the Uptake_{2}...
process was blocked by $10^{-4}$ M normetanephrine. Piperoxan (added to the bath 20 minutes before testing, which was performed without previous rinsing) in increasing doses caused parallel shifts in the log dose-response curves obtained with epinephrine (Fig. 3A) and norepinephrine (Fig. 3B). The effect of piperoxan on the phenylephrine-induced contraction was not constant. In some experiments, the phenylephrine response was not affected by antagonist concentrations as high as $3 \times 10^{-5}$ M; at higher concentrations (up to $5 \times 10^{-4}$ M) there was only a tendency to a reduced maximum response, indicating a nonspecific effect of piperoxan. In other experiments, increasing concentrations of piperoxan ($3 \times 10^{-7}$ to $5 \times 10^{-4}$ M) produced slight shifts of the phenylephrine dose-response curves toward higher doses (Fig. 3C). The reason for the inconsistency in the action of piperoxan is not known. It was observed that two vascular segments from the same cat tested simultaneously behaved similarly. No correlation was found whether or not the tests were carried out on sympathectomized vessels, in the presence of propranolol ($10^{-6}$ M) to block the beta receptors, in the presence of normetanephrine ($10^{-6}$ M) to inhibit the uptake mechanism, or in the presence of both propranolol and normetanephrine.

Piperoxan did not produce parallel shifts in the dose-response curves obtained with isoproterenol; instead, reductions in the maximum response and the slope were observed. Sometimes there was even a slight sensitization of the vessel for this catecholamine in the presence of piperoxan (Fig. 3D). After washout, the original effect of isoproterenol was restored, indicating a noncompetitive antagonism of the response. With the idea that the contractile effect of isoproterenol might be mediated by beta receptors, tests were performed with DL-INPEA and propranolol, which inhibit such receptors in a competitive manner (the vessels in these experiments had not been sympathectomized and the organ bath did not contain any pharmacologically active compound besides the beta antagonist to be tested). However, both DL-INPEA (Fig. 4) and propranolol ($10^{-11}$ to $5 \times 10^{-8}$ M) caused a decrease in the slope and the maximum of the dose-response curves, suggesting that the inhibitory effect on the isoproterenol-induced contraction was not specific. The nonspecificity of this inhibitory effect was confirmed by the finding that DL-INPEA (tested in concentrations of $10^{-8}$ and $10^{-7}$ M) also inhibited the contractile response to carbamylcholine ($10^{-6}$ to $10^{-5}$ M). The two beta-blocking agents did not have any contractile effect of their own in the doses tested.

**Dissociation Constant for Receptor-Antagonist Complex.**—Based on the reduction in sensitivity of a test system to various agonists occurring as a consequence of competitive antagonism, it is possible to calculate the dissociation constant for the receptor-antagonist complex ($K_B$) according to Eq. 4. For a particular antagonist acting on a given receptor, this constant should be the same irrespective of the agonist used. Eq. 4 can also be expressed in its logarithmic form, and the log (dose ratio - 1) plotted as a function of the negative logarithm of the concentration of the antagonist in the region of the receptors, according to Arunlakshana and Schild (27). should give a straight line with a slope of -1. Equation 5 illustrates the results obtained from experiments with norepinephrine and phenylephrine (i.e., those cases in which a parallel shift of the phenylephrine response did occur) using piperoxan as the antagonist. Regression analysis of the values from the norepinephrine tests showed a good linear correlation. The slope was $-0.90$ which, according to Student’s $t$-test, is not significantly different from $-1$ ($P > 0.05$). At the intercept of the straight line with the abscissa when the slope is unity, the value for $pA_2$ is equal to $pA_1$ (negative logarithm of that concentration of antagonist which mandates twice as high a concentration of agonist to elicit a given response) (27). The $pA_2$ value thus obtained for piperoxan with norepinephrine as the agonist was 7.06 (Fig. 5). The $K_B$ calculated from individual experimental values according to Eq. 4 was $1.24 \times 10^{-7} \pm 0.22 \times 10^{-7}$ M (mean ± SE).
ADRENERGIC RECEPTORS IN BRAIN VESSELS

FIGURE 3

Dose-response curves from single experiments with epinephrine (A), norepinephrine (B), phenylephrine (C), and isoproterenol (D) before and after addition of increasing doses of the alpha antagonist piperoxan. Piperoxan was administered 20 minutes before the experiment and was present in the organ bath when the cumulative dose-response curve was determined. Following washout and after the vessel had returned to its original tension, a new dose of piperoxan was added. All vessels were sympathectomized, and the stock solution for the organ bath contained 10⁻³M propranolol and 10⁻⁴M normetanephrine. Values from B and C are included in the plots of Figure 3.

Also the values from the phenylephrine experiments showed good linearity (Fig. 5), but the slope of the straight line (-0.52) was significantly different from -1 (P = 0.001). Therefore, the pA₂ intercept gives an erroneously high value for pA₂ and a correct Kᵣ value cannot be obtained, since all of the assumptions on which its determination is based are not valid.

Conduction by Phenylephrine Not Mediated by Alpha Receptors.—The straight line in the Arunlakshana-Schild plot, representing the changes in sensitivity of the test system to phenylephrine in the presence of piperoxan (Fig. 5), had a slope of less than unity. In other words, the displacement of the log phenylephrine dose-response curve was smaller than that expected from a competitive inhibition involving a simple bimolecular mechanism according to receptor theory. Similar observations have been made in other tissues and satisfactorily explained by interference from a saturable uptake mechanism for the agonist (17).

In the tests that were carried out on the feline middle cerebral artery, attempts were made to eliminate the neural uptake of amine (Uptake₁) either by sympathetic denervation or by cocaine and to block the extraneuronal uptake (Uptake₂) by normetanephrine. Therefore, we thought that the deviation from the prediction of receptor theory could involve a contractile action of phenylephrine not mediated by alpha receptors. This idea was
Effect of increasing concentrations of the beta antagonist DL-INPEA on the isoproterenol-induced contraction of the nonnervated feline middle cerebral artery. No additional drugs were present in the organ bath.

tested on arterial preparations to which phenylephrine was added in a cumulative way until concentrations beyond that giving a maximum response were reached. Dose-response curves were determined before and after partial irreversible blockade of the alpha receptors with phenoxybenzamine (10^-7M or 10^-6M for 30 minutes followed by washout before testing). It can be seen from the log concentration-response curves in Figure 6 that, following a plateau, the phenoxybenzamine-treated vessel showed a second contraction phase at high doses of phenylephrine to a level as high as that induced by the agonist before alpha blockade. When, on the other hand, the norepinephrine response was similarly tested after treatment with 10^-7M phenoxybenzamine (which reduced the effect by approximately 70%), no increased contraction occurred at agonist doses up to 3 x 10^-4M. These findings thus confirm the assumption that phenylephrine also acts on some site other than the alpha receptor.

Irreversible Competitive Antagonism.—The beta-haloalkylamines, phenoxybenzamine and dibenamine, are competitive antagonists which inactivate the alpha receptors and therefore produce an irreversible blockade (21, 28). The effect of phenoxybenzamine was tested on the contractile response to epinephrine, norepinephrine, phenylephrine, and isoproterenol. Graded blockade (examples are given in Fig. 7A and B) initially caused a parallel displacement of the log dose-response curves for epinephrine, norepinephrine, and phenylephrine followed by a decrease in maximum and slope. With isoproterenol, no such initial parallel shift was observed (Fig. 7C). The norepinephrine-induced contraction was also studied after blockade with dibenamine (Fig. 7D), which had principally the same effect as phenoxybenzamine.

Dissociation Constants for the Receptor-Agonist Complex.—The dissociation constant for the receptor-agonist complex (K_a) was calculated for norepinephrine using the log dose-response curves before and after partial irreversible blockade of the alpha receptors with phenoxybenzamine (21). The vessels were sympathectomized to abolish neuronal uptake of the catecholamine, and interference by any beta-receptor effect was avoided by the presence of 10^-8M propranolol in the buffer solution. Figure 8A shows an example from one experiment in which partial alpha-receptor blockade reduced the maximum response to norepinephrine by 70%. Values

\[
\log(\frac{[A]}{[A]_0})
\]

Arunalakshana-Schild plot based on five experiments with norepinephrine and five experiments with phenylephrine in the presence of increasing concentrations of piperoxan (one of the norepinephrine experiments and one of the phenylephrine experiments are illustrated in Fig. 8B and C, respectively), which reduced the sensitivity of the middle cerebral artery to the two agonists. Conditions are the same as those described for Figure 3. The pA_2 value is the negative logarithm of that concentration of the antagonist which mandates twice as high a concentration of the agonist to elicit a given response. The equations for the lines are given together with the correlation coefficient (r) and the number of values (n).
ADRENERGIC RECEPTORS IN BRAIN VESSELS

Contractile action of phenylephrine on a non-denervated feline middle cerebral artery in vitro. In the first test, no alpha antagonist was present; agonist concentrations were tested beyond $E_{am}$; 30 minutes before the next test $10^{-3}$M phenoxycbenzamine was added to the organ bath to abolish a fraction of the alpha receptors. After washout, a new cumulative application of phenylephrine was performed. Finally, the experiment was repeated after a more pronounced irreversible blockade of the alpha receptors with $10^{-4}$M phenoxycbenzamine. Note that the second contraction phase at high phenylephrine concentration reached the level of $E_{am}$ in spite of alpha receptor blockade.

were taken from such curves for the double reciprocal plot of the norepinephrine concentrations that produced the same response before ([A]) and after ([A'] receptor blockade; this plot was a straight line (Fig. 8B). The dissociation constant was calculated according to Eq. 2 and had a mean value of $1.73 \times 10^{-4} \pm 0.48 \times 10^{-4}$M for five experiments. The $K_A$ value obtained in the illustrated experiment ($2.17 \times 10^{-8}$M, Fig. 8B) was used in Eq. 3 to determine the fractional receptor occupancy ($[R_A]/[R_T]$), which was plotted as a function of norepinephrine dose (Fig. 8C, open circles). The curve obtained was compared with that representing a plot of relative actual response ($E/E_{am}$) as a function of norepinephrine dose (Fig. 8C, solid circles). This relation between relative actual response and receptor occupancy is shown in Figure 8D, where it can be seen that the norepinephrine response is not directly proportional to the fraction of receptors occupied, that half-maximum contraction occurs with only about 11% of the receptors occupied by norepinephrine, and that $E_{am}$ is reached at about 75% receptor occupation.

EXPERIMENTS ON ACTIVELY CONTRACTING ARTERIES

General Considerations.—There is evidence that the vascular smooth muscle in, for example, aortic strips (29) is almost completely relaxed in vitro and therefore does not allow the demonstration of a dilatory response. However, we have indications that the isolated feline middle cerebral artery in fact can relax under resting conditions to a slight degree in the presence of isoproterenol (Fig. 7C), terbutaline, acetylcholine and histamine (14) or when the temperature of the organ bath is reduced. Therefore, attempts were made to reveal a more clear-cut dilatory response by giving the vessel an initial tonic contraction induced by acetylcholine (carbamylcholine) or 5-hydroxytryptamine (12). To avoid interference from contractile effects mediated by the alpha receptors, it would be desirable to produce irreversible blockade of these receptors; however, the beta-haloalkylamines also block the effects of 5-hydroxytryptamine (30, 31) and acetylcholine (32, 33) on isolated smooth muscle preparations. For this reason, we determined the lowest dose and exposure time that would produce total alpha-receptor blockade (as revealed from full cumulative dose-response curves for norepinephrine) but would still allow a useful effect with the compound to be applied to induce active contraction. Such a situation could not be achieved with either acetylcholine or carbamylcholine in the way reported for preparations such as guinea pig tracheal strips (22). On the other hand, exposure for 30 minutes to $10^{-5}$M dibenamine, which completely blocked the alpha receptors (tested with norepinephrine), still left a contraction of 200-500 dynes with $3 \times 10^{-6}$M 5-hydroxytryptamine. The contraction remained at a steady level for at least 15 minutes, during which each of the various amines to be tested was added by cumulative application. Angiotensin, at concentrations in the bath up to 0.03 mg/ml, did not give more than a faint contraction even in the non-blocked middle cerebral artery.

Relative Potency.—The ability to produce a dilation (relaxation) of the feline middle cerebral artery was tested under the conditions outlined in the preceding section. Although dibenamine blocks Uptake, to some extent (18), a more complete inhibition was achieved by either sympathectomy or constant exposure of the vessel to $10^{-4}$M cocaine. The stock buffer solution also contained $10^{-4}$M normetanephrine to block Uptake. The relative potency (comparison of mean ED$_{50}$ values) for...
dilating the preparation was isoproterenol > norepinephrine > epinephrine > terbutaline (Table 2). The effects were dose dependent (Fig. 9); the mean maximum responses are given in Table 2. Iso- proterenol was 2,500 times more potent than terbutaline. The potency ratio of norepinephrine to epinephrine was 1.2:1.

Reversible Competitive Antagonism and $K_a$ Value.—No irreversible competitive antagonist is available for beta receptors. The two reversible antagonists, propranolol (Fig. 10A) and DL-INPEA ($3 \times 10^{-8}$M), lowered the sensitivity of the test system to terbutaline without changing the slope or $E_{\text{max}}$ of the log dose-response curve. Since terbutaline in the doses tested did not contract the middle cerebral artery, there was no reason to treat the vessels with dibenamine. The values for the dose ratio minus one and the concentration of propranolol (four experiments) plotted against each other in their logarithmic form according to Arunlakshana-Schild (27) fit a straight line with a slope of $-0.92$ (Fig. 11), which is not different from unity (Student's $t$-test, $P > 0.05$). This finding confirmed that the inhibition of the terbutaline-induced dilation by propranolol was competitive, involving a bimolecular process according to receptor theory. The mean value for $pA_2$ at the intercept with the abscissa, was 8.78. The dissociation con-
Representative experiment demonstrating the quantitative aspects of the contractile effect of norepinephrine on the feline middle cerebral artery. The artery had been sympathetically denervated beforehand and was exposed to 10^{-6} M propranolol throughout the experiment. A: Response before and after irreversible blockade of a fraction of alpha receptors by 10^{-6} M phenoxybenzamine (exposure for 30 minutes followed by a washing period before testing of the agonist). B: From the curves obtained in A a double reciprocal plot was made and the dissociation constant \( K_a \) calculated. C: The relative response (solid circles) was obtained from the noninhibited curve in A and plotted together with the fractional receptor occupancy (open circles) as a function of agonist dose. D: Relation between actual response and fractional receptor occupancy determined from the curves in C.

### TABLE 2

Dilatory Effects of Sympathomimetic Compounds on the Middle Cerebral Artery after a Tonic Contraction of Up to 500 dynes had been Induced with \( 3 \times 10^{-8} \) M 5-Hydroxytryptamine

<table>
<thead>
<tr>
<th>Compound</th>
<th>( N )</th>
<th>( E_{am} ) (dynes)</th>
<th>( ED_{50} ) (m)</th>
<th>Relative potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoproterenol</td>
<td>13</td>
<td>116 ± 20</td>
<td>(1.26 ± 0.63) \times 10^{-4}</td>
<td>1</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>5</td>
<td>65 ± 23</td>
<td>(3.14 ± 1.29) \times 10^{-4}</td>
<td>0.004</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>5</td>
<td>107 ± 27</td>
<td>(3.87 ± 1.72) \times 10^{-4}</td>
<td>0.003</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>13</td>
<td>170 ± 30</td>
<td>(3.15 ± 0.30) \times 10^{-4}</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

Results are means ± se. The vessels were either sympathectomized 1 week beforehand or constantly exposed to 10^{-6} M cocaine during testing. The stock buffer solution also contained 10^{-6} M normetanephrine to block any extraneuronal uptake. Relative potency was based on a comparison of \( ED_{50} \) values, assuming the value for isoproterenol to be unity. \( N \) - number of experiments, \( E_{am} \) - maximum dilation, and \( ED_{50} \) - dose producing a half-maximum dilation.
Agonist concentration

**FIGURE 9**

Dilatory response to isoproterenol, epinephrine, norepinephrine, and terbutaline obtained in a sympathectomized middle cerebral artery given a tonic contraction of 450 dynes by $3 \times 10^{-4} \text{M}$ 5-hydroxytryptamine. The alpha receptors were blocked by exposure of the vessel to $10^{-4} \text{M}$ dibenamine 30 minutes before testing, and, after washing, 5-hydroxytryptamine was administered to produce the tonic contraction. Each agonist was then added and a full cumulative dose-response curve was determined. Following rinsing, the vessel was allowed to relax to its original tension, 5-hydroxytryptamine was again added, and another agonist was tested. Uptake was inhibited with $10^{-4} \text{M}$ normetanephrine in the stock buffer solution.

stan for the receptor-antagonist (propranolol) complex was calculated according to Eq. 4, using the individual values from the four experiments; it had a mean value of $2.31 \times 10^{-9} \pm 0.50 \times 10^{-9} \text{M}$.

Propranolol also shifted the log dose-response curves for isoproterenol toward higher concentrations (Fig. 10B). The Arunlakshana-Schild plot (Fig. 11) showed a good linear correlation and the slope was $-1.27$ which, according to Student's $t$-test, is not different from unity ($P > 0.05$). The $pA_2$ at the intercept with the abscissa was 9.17. The mean $K_0$ value, according to Eq. 4 (five experiments), was $1.77 \times 10^{-9} \pm 0.77 \times 10^{-9} \text{M}$, which is not significantly different ($P > 0.05$) from the corresponding value obtained with terbutaline as the agonist.

**Discussion**

The order of potency by which the various amines tested produced a constriction of the vascular preparations indicated that the response involved alpha receptors (22, 34). The parallel shift of the dose-response curves toward higher concentrations of norepinephrine, epinephrine, and phenylephrine seen in the presence of increasing levels of the alpha-receptor antagonist, piperoxan (26, 35) confirmed this hypothesis. The competitive nature

**FIGURE 10**

Dilatory effects of terbutaline (A) and isoproterenol (B) on a nonadenverated middle cerebral artery given an active tone by $3 \times 10^{-4} \text{M}$ 5-hydroxytryptamine. The normal curves were determined and the preparation rinsed, then propranolol was injected into the organ bath 20 minutes before the next determination, which started when 5-hydroxytryptamine was administered to give a steady level of contraction. The agonist was tested in the presence of the antagonist. After a washing period, the experiment was repeated with the next dose of antagonist. The buffer stock solution contained $10^{-4} \text{M}$ cocaine. In the experiments with isoproterenol (B), the buffer also contained $10^{-4} \text{M}$ normetanephrine and the alpha receptors were initially inhibited by 30 minutes of exposure to $10^{-4} \text{M}$ dibenamine. Values from both sets of curves are included in Figure 11.
ADRENERGIC RECEPTORS IN BRAIN VESSELS

For this reason it was not possible to calculate accurate pA₂ or Kₐ values with phenylephrine as the agonist. Using norepinephrine, the Kₐ for the alpha receptor-piperoxan complex was 1.24 × 10⁻⁵M and pA₂ was 7.06. The pA₂ and Kₐ values are important parameters in the characterization of a receptor, because they should be the same irrespective of the agonist used for a particular antagonist acting on a specific receptor: also, for a given antagonist acting on the same type of receptor, they should be equal for various tissues (39). The presently obtained value of pA₂ for the feline middle cerebral artery is lower (and hence the Kₐ higher) than that for the ovarian follicular wall tested under similar conditions (40), and the pA₂ value is higher (Kₐ lower) than that reported for the vas deferens of the rat and the intestine of the rabbit, although in the latter study (41) the precautions necessary for quantitative analysis of adrenoceptors were not taken.

These discrepancies together with the high ED₆₀ values and the finding that the specific alpha stimulant, phenylephrine, only acted as a partial agonist (being less potent than isoproterenol and having a potency ratio to norepinephrine about ten times lower (17) than it is in other tissues) suggest the presence of an unusual type of alpha receptor in pial arteries.

Isoproterenol contracted the middle cerebral artery but only at high doses. There is now reason to believe that this action is not mediated by beta receptors as previously assumed (12) and that the inhibitory effect of certain beta antagonists on cerebral vasoconstriction is nonspecific (42-44). On the other hand, it is not yet clear whether the isoproterenol contraction is an alpha-adrenergic effect; although the antagonism by piperoxan was reversible, it appeared to be noncompetitive.

The competitive alpha antagonists, dibenamine and phenoxybenzamine, are for practical purposes irreversible because of the very slow recovery following inactivation of the receptor (28). Based on the contractile responses before and after inactivation of a fraction of the receptors with phenoxybenzamine, it was possible to quantify (21) the effects of the sympathetic transmitter, norepinephrine, on the alpha receptors in pial vessels. The dissociation constant (Kₐ) was 1.73 × 10⁻⁹M, which means that the affinity of norepinephrine for the receptors is similar to that found for the rat portal vein (24) but lower than that found for the rabbit aortic strip (17). Furthermore, the relation between the actual response obtained on the middle cerebral artery with norepinephrine and the alpha-
receptor occupancy showed that the contraction was not directly proportional to the fraction of receptors occupied; the initial parallel shift obtained in the dose-response curves after irreversible blockade of the alpha receptors with β-haloalkylamines also suggests this conclusion (21). In fact, half-maximum contraction was achieved with only about 11% of the receptors occupied, and maximum contraction was reached when about 75% were occupied by norepinephrine.

The reciprocal ability of the sympathomimetic compounds to dilate (relax) the middle cerebral artery and the competitive inhibition of the response produced by INPEA and by propranolol (further tested with the Arunlakshana-Schild plot) show that the dilatory response is mediated by beta receptors (17, 22). On the basis of differences in potency rank order for beta-sympathomimetic effects on various tissues, it has been possible to separate responses mediated by beta, receptors (e.g., in the heart) and those mediated by beta, receptors (e.g., in the lung); the literature has been reviewed by Furchgott (17) and Persson (45). The ratio between the ED₅₀ values for the dilatory effects of norepinephrine and epinephrine agreed with a beta, type of action (17). This hypothesis was confirmed in a more direct way by comparing the potency of isoproterenol with that of the beta agonist terbutaline (46); isoproterenol was 2,500 times more active. The beta type receptors in the pial arteries are thus different from those in the peripheral circulation, since relaxation of the rabbit thoracic aorta (22), reduction in vascular resistance of cats (46), and vasodepression effects tested in dogs (47) appear to be mediated by beta, receptors. Since irreversible competitive antagonists are not available for the beta receptors, it was not possible to further characterize the reaction between this receptor and the agonists.

The interaction between the reversible competitive antagonist, propranolol, and the beta receptor seemed to follow a simple bimolecular process according to receptor theory, as revealed in the Arunlakshana-Schild plot using terbutaline and isoproterenol as agonists. The αₐ values, 8.78 and 9.17, agreed with those reported for other isolated tissues (17), and the Kᵦ values obtained with the two agonists were not significantly different from each other.

In conclusion, the pial arteries possess both alpha and beta receptors mediating vasoconstriction and dilation, respectively, as tested on the isolated feline middle cerebral artery. The reaction with the alpha receptors shows certain features that are different from those expected for a usual type of alpha receptor. The beta receptor can be classified as a beta₁ type in contrast to beta receptors in the peripheral vascular bed (with the exception of the coronary circulation), which have been designated as beta₂ receptors.

Acknowledgment

This paper is dedicated to our friend and co-worker Kai C. Nielsen (1920-1973), who was Head of Neurosurgical Clinic A, University Hospital of Lund. We wish to express our sincere gratitude to Professor Robert F. Furchgott, Department of Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, New York, for stimulating discussion, valuable criticism and helpful advice.

References


Circulation Research, Vol. 33, December 1974

Circulation Research, Vol. 35, December 1974
Pharmacological Characterization of Adrenergic Alpha and Beta Receptors Mediating the Vasomotor Responses of Cerebral Arteries In Vitro
LARS EDVINSSON and CHRISTER OWMAN

Circ Res. 1974;35:835-849
doi: 10.1161/01.RES.35.6.835

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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