Cerebral arterial spasm

Part 2: *In vitro* contractile activity of serotonin in human serum and CSF on the canine basilar artery, and its blockage by methylsergide and phenoxybenzamine

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*In vitro* experiments were performed to determine the contractile activity of human serum and cerebrospinal fluid on the canine basilar artery. The majority of contractile activity in these CSF samples, which were collected 2 to 7 days following a subarachnoid hemorrhage, was proven to be due to serotonin. Serotonin was capable of producing a prolonged contraction of the artery depending on its activity. Methylsergide reversibly blocked the artery’s response to serotonin and caused a contraction of the basilar artery. Phenoxybenzamine irreversibly blocked the basilar artery’s response to serotonin, serum, and CSF.

**KEY WORDS** — cerebral arterial spasm • vasospasm • serotonin • cerebrospinal fluid • subarachnoid hemorrhage • basilar artery • methylsergide • phenoxybenzamine

Part 1 of this study (pp. 433-441)² demonstrated that serotonin is the likely agent in blood responsible for the cerebral arterial spasm that follows a subarachnoid hemorrhage. This paper reports further experiments using the same *in vitro* method to demonstrate that: 1) serotonin in physiological concentrations will produce prolonged contractions of the canine basilar artery, 2) the great majority of contractile activity present in the cerebrospinal fluid (CSF) of three patients following subarachnoid hemorrhage is due to serotonin; and 3) that phenoxybenzamine will block the artery’s response to serotonin.

**Materials and Methods**

*Chamber Studies*

Ten dogs of both sexes, weighing between 19 and 26 kg, were used for these experiments. The procedures for obtaining basilar artery segments and the *in vitro* techniques used were the same as those reported previously.² To avoid foaming when solutions containing protein were
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added, a rotating magnetic stirring bar was used in the chamber.

Blood and Serum Studies

Blood from dogs and human volunteers was obtained by intravenous puncture with an 18-gauge needle and collected in a plastic syringe. It was allowed to clot in centrifuge tubes for 1 hour at room temperature and then the clot was sedimented in a clinical centrifuge for 10 minutes. The serum was removed by aspiration, centrifuged again for 10 minutes, and then kept at 0°C until its contractile activity was tested.

Human Cerebrospinal Fluid Studies

Cerebrospinal fluid was obtained by lumbar puncture with a 20-gauge needle. The first 2 to 3 ml were allowed to escape before a collection of 10 to 25 ml was begun. No puncture showed evidence of fresh blood. The samples were immediately cooled in ice and stored frozen. Freezing for up to 2 weeks had no effect on the contractile activity of the fluid.

Chromatography was done with a separate strip of Whatman 3MM paper* for each sample in a descending fashion for 8 hours. Solvent systems used were butanol: acetic acid: water, 4:1:1 (Solvent 1) and butanol: pyridine: water, 1:1:1 (Solvent 2). The papers were dried in a 40°C forced air oven and the samples eluted from the paper with dilute HCl by capillary flow for 3 hours. The eluates were lyophilized and dissolved in 0.5 ml of water for testing in the chamber to determine if serotonin was present in the CSF.

Compounds Used

The water for all solutions was triply distilled from glass containers. The chemicals used to make up the Krebs-Ringer buffer and the chromatography solution were all American Chemical Society certified reagent grade. The vasoactive and blocking agents were made up fresh each day in water, except for prostaglandin F₂α,* which was made up as a concentrated stock solution and stored at 4°C. The epinephrine solutions were 10 μM in Na₂EDTA to prevent trace metal catalyzed oxidation.

Results

Serotonin-Induced Contractions

All arterial segments responded to serotonin in a similar manner as shown in Fig. 1. For these 10 arterial samples the mean K_{ED50} was 6.3 ± 0.9 × 10⁻⁹ M (± standard error of the mean, SEM) and the mean C_{MAX} was 8.0 ± 0.7 gm (± SEM). The range of K_{ED50} for serotonin was 1.5 - 9.5 × 10⁻⁹ and the range of C_{MAX} was 5.0 to 11.9 gm. Studies were conducted on three arterial segments to determine if serotonin would cause a prolonged contraction of the basilar artery in the chamber. A concentration of serotonin, just sufficient to give a maximal contraction, was added to the chamber and left there for 2 hours. During the first 30 minutes, the contraction slowly decreased to approximately 50% of maximal tension where it stayed for the remaining 2 hours. The arteries were then washed in the usual manner, after which they promptly returned to their normal baseline tension. Next, serotonin just sufficient to produce 50% of the maximal contraction was added to the chamber and again left there for 2 hours; the arteries maintained a 50% maximal contraction for the entire 2 hours and gave no signs of a lessened response. Again the arteries were

*Serotonin (5HT) (serotonin creatinine sulfate) and epinephrine (Epi) (L-epinephrine-bitartrate) were obtained from the Sigma Chemical Company, P. O. Box 14508, St. Louis, Missouri 63178. Methylsergide (MS) was a gift from the Sandoz Pharmaceutical Company, Division of Sandoz-Wander, Inc., E. Hanover, New Jersey 07936. Prostaglandin F₂α (F₂α) (tromethamine salt) was a gift from the Upjohn Company, Kalamazoo, Michigan 49001, and phenoxybenzamine (Phb) (phenoxybenzamine hydrochloride) was a gift from Smith, Kline & French Laboratories, Philadelphia, Pennsylvania 19101.

*K_{ED50} = concentration of an agent at which 50% of the maximal contraction obtainable with that agent occurs.

C_{MAX} = maximal contraction obtainable with the agent.
TABLE 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Serum (ng/ml)</th>
<th>Molar</th>
</tr>
</thead>
<tbody>
<tr>
<td>human 1</td>
<td>480</td>
<td>$2.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>human 2</td>
<td>840</td>
<td>$4.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>human 3</td>
<td>120</td>
<td>$0.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>dog</td>
<td>360</td>
<td>$2.0 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

* Determined by canine basilar artery contraction.

Fig. 1. Mean cumulative log dose-response curve of the canine basilar artery for serotonin. N = number of preparations each obtained from a different dog. Brackets = standard error of the mean (SEM). 100% response to serotonin = 8.0 gm ± 0.7 SEM.

washed in the usual manner and they promptly returned to their baseline tension.

Blood Experiments

Serum was added to the chamber in volumes not exceeding 500 μl and cumulative dose-dependent contractions were obtained (Fig. 2). Four arterial segments were tested with serum from three human volunteers and one dog. The serum concentrations of serotonin, assuming all activity was due to serotonin, are given in Table 1. These values were calculated from a dose-response curve of pure serotonin obtained on the same arterial preparation. The values fell within the range of serum serotonin values reported in the literature. In an attempt to simulate blood being released into the CSF, 1 ml of a human volunteer's fresh blood was mixed with 4 ml of Krebs-Ringer buffer at 37°C and maintained at that temperature for 30 minutes. This solution was then tested in the chamber and found to contain twice as much serotonin as did the serum from 1 ml of the same blood sample. It was observed that those arteries that did not respond to epinephrine responded to serum just as well as those arteries that were sensitive to epinephrine. It was concluded from this that epinephrine was not responsible for the contractile activity of serum.

Fig. 2. Polygraph tracing of the isometric contraction of a basilar artery segment to cumulative additions of human serum. Numbers at the base of the tracing indicate the grams of tension at full scale reading, and the arrows at the base of the tracing show when this was changed. Arrows on the tracing indicate when serum was added in the volumes shown.

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Effects of Methylsergide and Phenoxybenzamine on Serotonin, Serum, And F₂α-Induced Contractions

Methylsergide has been found to block the response to serotonin in other smooth muscle preparations.⁵ Therefore, experiments were conducted with two arterial segments to determine if methylsergide would block the response of the basilar artery to serotonin. Methylsergide was found to give a dose-dependent contraction of its own, with a maximal contraction of 58% of the maximum obtained with serotonin, and a $K_{ED50}$ of $9.0 \times 10^{-8}$ M. When methylsergide was added to the chamber to give a final concentration of $10^{-4}$ M and then serotonin or serum was added, no additional contraction above that produced by the methylsergide was obtained, although the concentration of serum or serotonin added was sufficient to elicit a greater contraction in the absence of methylsergide (Fig. 3). In contrast, when $F₂α$ was added after the methylsergide, a further contraction to the level expected without methylsergide was obtained (Fig. 3). Methylsergide blockage of serotonin was completely eliminated by the usual washing procedure. These results established the fact that $F₂α$ is not responsible for the contractile activity of serum. The similar action of this blocking agent on the contractile response of these arteries to both serum and serotonin confirms the identity of serotonin as the active agent in serum.

Phenoxybenzamine has been shown to block or reverse the spasm following the subarachnoid injection of blood.⁶⁻⁹ Experiments were therefore conducted with phenoxybenzamine to determine if it would block the response of the basilar artery to serotonin. Phenoxybenzamine at a final concentration of $10^{-4}$ M, when allowed to remain in contact with three different arterial segments for 30 minutes, effectively blocked the arterial response to serotonin, and concentrations of serotonin up to $5 \times 10^{-6}$ M would not produce contractions. This was an irreversible blockage, and even after repeated washings for 4 hours the arteries remained totally unresponsive to serotonin although they were responsive to prostaglandin $F₂α$. Another experiment was performed to determine if phenoxybenzamine was effective in the presence of serotonin. Serotonin was first added to the chamber in a final concentration of $10^{-7}$ M and the artery allowed to reach its maximal contraction. Phenoxybenzamine, (final concentration $10^{-4}$ M) was then added and both agents allowed to remain in the chamber for 30 minutes (Fig. 4). During this 30 minutes the artery returned essentially to its baseline-resting tension, and, after washing the artery in the usual manner, serotonin would not give a contraction at any concentration tested. Four arterial segments, after treatment with $10^{-4}$ M

![Fig. 3. Polygraph tracing of the isometric contraction of a basilar artery segment to the addition of methylsergide (MS) and the subsequent additions of serotonin, human serum, and $F₂α$. Numbers at the base of the tracing indicate the grams of tension at full scale reading and the arrows at the base of the tracing show when this was changed. Arrows on the tracing indicate when compounds were added in the volumes or final molar chamber concentrations shown.]
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Fig. 4. Polygraph tracing of the isometric contraction of a basilar artery segment. The chamber fluid was made $10^{-4} \text{ M}$ in serotonin and reached a stable state of contraction as shown at the left of the tracing. Phenoxybenzamine was then added to reach a final concentration of $10^{-5} \text{ M}$ as shown on the tracing. Numbers at the base of the tracing indicate the grams of tension at full scale reading and the arrows at the base of the tracing show when this was changed. The time course of the tracing was changed and the scales are shown at the bottom of the tracing.

Phenoxybenzamine for 30 minutes, would not give a contraction when dog or human serum was added. They would react to $F_2\alpha$ with some contraction at even the lowest concentration, but the $C_{\text{MAX}}$ was reduced by 50% and the $K_{E50}$ shifted by a factor of 100 to higher concentrations for the $F_2\alpha$ cumulative dose-response curves. It was concluded from these results that phenoxybenzamine will block the artery's response to serotonin. The blockage is irreversible in vitro and is not prevented by physiological concentrations of serotonin. Phenoxybenzamine will also block the contractile activity of serum, which further substantiates the premise that such activity is due to serotonin.

**Human Cerebrospinal Fluid Studies**

The basilar artery preparation was then used as an assay system for determining if there was contractile activity in the CSF of patients who had suffered a subarachnoid hemorrhage from an aneurysm. Control CSF was obtained from two patients undergoing lumbar myelography for discogenic disease. This CSF, when added to the chamber in amounts up to 400 $\mu\text{l}$, caused no contraction of the basilar artery segment. CSF from three patients (Cases 1-3), who had documented subarachnoid hemorrhages 2, 5, and 7 days respectively prior to obtaining the CSF, caused dose-dependent contractions of three different arterial segments when added to the chamber in volumes of 50 to 500 $\mu\text{l}$. Figure 5 is a polygraph tracing showing the response obtained when 300 $\mu\text{l}$ of CSF from the patient in Case 2 was added to the chamber. When 500 $\mu\text{l}$ of CSF from the patient in Case 3 (7 days following hemorrhage) was added, almost 50% of the maximum serotonin contraction occurred. CSF samples from all three patients in the volumes added caused over 1.5 gm of contraction. The CSF in all three cases was clear xanthochromic fluid without evidence of fresh blood. One arterial segment was then treated with phenoxybenzamine as described above, and after such treatment 500 $\mu\text{l}$ of CSF from the patient in Case 3, given in a similar manner, would not cause a contraction.

The above results had suggested that serotonin was the agent in these CSF samples responsible for the contractile activity. To prove this, serotonin was isolated from two of these CSF samples (Cases 2 and 3) using paper chromatography. After lyophilizing, methanol extraction was done on these two CSF samples and the extracts chromatographed in Solvents 1 and 2 (see Methods and Materials). In addition, the CSF from Case 3 was applied directly to the paper and chromatographed in both solvent systems. Pure serotonin was also added to separate aliquots of each of the CSF samples and $R_f$ values* for serotonin

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*The $R_f$ values experimentally determined for Solvents 1 and 2 were: 0.38 and 0.73 respectively. An $R_f$ value of 0.70 for serotonin using Solvent 2 has been reported in the literature. $R_f = \frac{\text{distance compound migrates}}{\text{distance solvent migrates}}$. 

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![Graph showing isometric contraction of a basilar artery segment after addition of human CSF obtained 5 days after subarachnoid hemorrhage. Numbers at the base of the tracing indicate the grams of tension at full scale reading and the arrows at the base of the tracing show when this was changed. The arrow on the tracing indicates when the CSF was added in the volume shown.](image)

**Table 2**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Days Post Hemorrhage</th>
<th>CSF Serotonin (ng/ml)</th>
<th>Molar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>7</td>
<td>$4.1 \times 10^{-8}$</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5</td>
<td>$2.9 \times 10^{-8}$</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>15</td>
<td>$8.6 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

* Determined by canine basilar artery contraction.

This is not the first report on the use of an *in vitro* smooth muscle preparation to assay for contractile activity in blood or CSF obtained from patients following a subarachnoid hemorrhage. Buckell used the rat's stomach to assay “hematoma material taken from the immediate environment of a ruptured aneurysm.” He did report finding serotonin in these samples, but since this fluid contained “a mixture of old fluid blood with varying amounts of clot, cerebrospinal fluid, and flecks of necrotic brain tissue,” care must be taken in interpreting the results. Wilkins, et al., more recently used the rabbit aorta as an assay system and reported contractile activity in the CSF from patients following a subarachnoid hemorrhage. He did not identify the agent in CSF responsible for this activity.

The present *in vitro* method is well suited for studying cerebral arterial spasm since it employs segments of the arteries affected by spasm. The small chamber allows the testing of small volumes of agents or biological fluids. By limiting the additions to 5% of the...
volume of the chamber contents, electrolyte concentrations, pH, and temperature were not changed significantly. It is recognized that such an in vitro method, however reproducible, should be checked by in vivo experiments, if possible, before any definitive conclusions are reached. Such an in vivo study constitutes the third part of this study.\(^1\)

The data show that all canine basilar arteries respond in a consistent manner to additions of serotonin, but the range of \(K_{ED50}\) values demonstrates that there is almost a tenfold variation among the animals in their artery's sensitivity to serotonin. The sigmoidal shape of the dose-response curve (Fig. 1) with its very steep vertical portion is common to many physiological systems. Another example is the oxygen-hemoglobin association curve.\(^13\) This type of response allows small changes in the concentration of serotonin surrounding the arteries to cause large changes in the amount of contraction of the arteries. It is significant that all three CSF samples, collected 2 to 7 days following a subarachnoid hemorrhage, had serotonin concentrations that lie on the vertical portion of this curve.

It seems likely that serotonin is not altered in any way but is simply bound at its arterial receptor, since one addition of serotonin will keep the artery at a constant state of contraction for 2 hours. There is a fairly rapid rate of exchange between receptor-bound serotonin and serotonin free in solution as shown by both the rapid attainment of a constant state of contraction and the prompt relaxation of the arterial segments following removal of serotonin from the bathing fluid. The amount of contraction is proportional to the concentration of free serotonin in the medium surrounding the artery. The contraction induced by serotonin will probably last as long as serotonin remains in the surrounding medium. The data presented in this report show it will last at least 2 hours in vitro. The observation that, in vitro, the artery will maintain only a 50% maximal contraction for long periods of time may be related to the artery's inability metabolically to provide, in vitro, sufficient energy to maintain a greater contraction.

Serotonin is known to be released from platelets when blood coagulates.\(^15\) Most, if not all, of the contractile activity of serum was shown to be due to serotonin. In addition, serotonin was shown to be released from the platelets when fresh blood was diluted with Krebs-Ringer buffer and maintained at body temperature, a condition that simulates blood being released into the CSF. It is reasonable to believe, therefore, that serotonin is released from platelets into the CSF after a subarachnoid hemorrhage. To prove this, contractile activity was demonstrated in the CSF obtained from patients 2 to 7 days following a subarachnoid hemorrhage. The majority of this activity was shown by paper chromatography in two solvent systems to be due to serotonin. Furthermore, all of this activity was blocked by treatment of the arterial segment with phenoxybenzamine. No contractile activity was present in two control samples of CSF and a sample of CSF obtained 1 month following a subarachnoid hemorrhage. These data conclusively show that serotonin plays a major role in the physiological production of spasm. Supporting this is the recent finding by Zervas, et al.,\(^19\) that blood from dogs treated with reserpine is incapable of producing spasm in the dog. Serotonin was not present in this blood.

Phenoxybenzamine has been shown by several investigators\(^6-9\) to block or reverse the spasm of the large cerebral arteries following the subarachnoid injection of blood. It is stated by these investigators that it is phenoxybenzamine's blockage at the alpha adrenergic receptor that is responsible for its effect on spasm. The data presented here show that phenoxybenzamine blocks the response of the canine basilar artery to serotonin in vitro. This has been shown for many other smooth muscle preparations.\(^11\)

The present study contains the following evidence to support the conclusion that the alpha adrenergic receptor and the serotonin receptor are separate entities: 1) several arteries were observed that would not respond to either epinephrine or norepinephrine yet responded normally to serotonin; and 2) catacholamines were incapable of giving more than 50% of the maximal serotonin response regardless of the concentration added. In addition, Furch-
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gott\textsuperscript{10} has shown in the rabbit aorta that the alpha adrenergic receptor and the serotonin receptor are different. Further work is in progress to answer this question in more detail with respect to the cerebral arterial receptors.

Phenoxybenzamine and other haloalkylamines have been shown to react with sulphydryl groups in biological material\textsuperscript{18} via an ethylenimmonium intermediate to form a covalent bond. It has also been reported that the alpha adrenergic receptor is probably a protein.\textsuperscript{14} It is not surprising then, that phenoxybenzamine would block both the serotonin and the alpha adrenergic receptors and, in addition, partially block the artery’s response to prostaglandin F\textsubscript{2\alpha} since it is possible that phenoxybenzamine reacts with sulphydryl groups of several different receptors. While \textit{in vitro} this covalently bound inhibition is irreversible, it is reasonable to expect that \textit{in vivo} the blockage would last until the receptor proteins are replaced with new proteins. Thus, the turnover rate of the arterial receptor proteins would be the factor that determines how long the blockage lasts. Clinically, such blocking agents might be advantageous in treating spasm since they might be effective for several days after one administration. It should be noted that when using agents of this type the solutions should be made up \textit{fresh} just prior to use, as the reactive intermediate responsible for the alkylation of the receptors may not be stable.

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References


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