SUMMARY  Sustained contraction of isolated canine basilar artery was induced by addition of prostaglandin F₂₀, prostaglandin E₂, hemoglobin-containing solution, or serum. Human platelet aggregation was induced by 1.0 μM adenosine diphosphate. The sustained contraction of basilar artery and the aggregation of platelet, particularly its secondary aggregation, were inhibited in a dose-dependent manner by addition of chlorpromazine or amitriptyline; calmodulin antagonist. The molar concentrations at 50% inhibition by chlorpromazine or amitriptyline were 5.9 to 7.7 μM or 28 to 39 μM for the contraction of basilar artery and 57 μM or 111 μM for the secondary aggregation of platelet. The results were discussed mainly on the basis of interaction of psychotropic drugs and Ca²⁺, calmodulin-dependent enzymes, particularly myosin light chain phosphorylation.

AS YET THERE is no effective treatment of cerebral arterial spasm, and this may be a consequence of the event being multifactorially mediated. In a recent experimental delayed cerebral vasospasm, prostacyclin and prostaglandin F₂₀ (PGF₂₀) formation in the canine basilar artery has been significantly decreased while prostaglandin E₂ (PGE₂) synthesis has been significantly increased, suggesting that prostaglandins are involved in the pathogenesis of delayed cerebral vasospasm. In addition, a disproportionate formation of thromboxane A₂, main prostaglandin product of platelet, has been reported to play an important role in the evolution of delayed cerebral vasospasm. However, an intravenous infusion of prostacyclin or thromboxane synthetase inhibitor fails to reverse the delayed cerebral vasospasm. Recently, an intravenous administration of aminophylline or nifedipine or an intravenous bolus injection of papaverine has failed to reverse the delayed cerebral vasospasm. If the delayed cerebral vasospasm is a multifactorially mediated process, the use of selective antagonists to individual agents would theoretically be of limited value, and a derangement of the contractile process of actin-myosin excitation contraction coupling in the vascular smooth muscle could be one of the important processes in the delayed cerebral vasospasm. In addition, neurological deterioration associated with subarachnoid hemorrhage could be partly due to embolization of platelet thrombi.

Recent studies have indicated that calmodulin is an important functional protein in the contraction of smooth muscle and the secondary aggregation of platelet, and that psychotropic drugs such as phenothiazine and tricyclic antidepressants inhibit the function of calmodulin. Consequently, calmodulin antagonists could be an ideal agent for the treatment of delayed cerebral vasospasm. The present study examines the effect of chlorpromazine or amitriptyline on the contraction of isolated canine basilar artery induced by PGF₂₀, PGE₂, hemoglobin (Hb)-containing solution, or serum, currently available as in vitro model of cerebral vasospasm as well as on the human platelet aggregation induced by adenosine diphosphate (ADP).

Materials and Methods

Relaxation Studies of Isolated Canine Basilar Artery

Adult dogs, 10 to 18 kg in weight, were sedated with an intravenous administration of 50 mg/kg of sodium pentobarbital and sacrificed by a rapid exsanguination. The brain with the basilar artery attached was removed rapidly. A 4 mm long segment of the basilar artery was made and then mounted on rigid parallel prongs in a chamber described previously. The chamber was filled with 8 ml of modified Krebs solution of the following composition: NaCl 118.9 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM, CaCl₂ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 14.9 mM, and dextrose 5.6 mM (pH 7.4), aerated with 95% O₂ and 5% CO₂, and warmed at 37 ± 0.5°C by means of a circulating temperature bath.

Isometric tension of the arterial segment in vitro was measured with a Nihon-Koden PD transducer (NihonKoden Kogyo Co., Tokyo, Japan). The arterial segment was allowed to stabilize at a resting tension of 200 to 400 mg for 1 hr and then increased to a resting tension of 3 gm before the start of the experiment. A trial contraction was made with a depolarizing solution at the beginning of each experiment to determine the condition of the arterial segment, and only those segments producing at least a 2-gm tension with the depolarizing solution were used. The depolarizing solution was composed of 76 mM K₂SO₄, 10 mM KCl, 16 mM KHCO₃, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM K₂HPO₄, and 5.6 mM dextrose.

Materials used for inducing the contraction of basilar artery in vitro were PGF₂₀, PGE₂, Hb-containing solution, and serum, the concentrations of which were 10⁻³ M, 10⁻⁵ M, 1 gm/dl, and 100 μl/ml, respectively, and induced the maximal contractions. Hb-containing solution and serum were made by the method of Toda et al. The content of Hb was assayed with a spectrophotometer (Hitachi Co., Tokyo, Japan) at a wavelength of 541 nm by the Hb-cyanide method. Methemoglobin was not detected in Hb-containing solution by the method of Van Assendelft. After in-
ducing the contraction of basilar artery, cumulative dose-response was obtained by increasing the concentrations of chlorpromazine or amitriptyline by a factor of about 3 while the previous dose remained in contact with the basilar artery and showed a steady response.30 At the end of each experiment, $1 \times 10^{-4}$ M papaverine was added and the relaxation induced by papaverine was taken as 100%.31 Only one cumulative dose-relaxation response to chlorpromazine or amitriptyline was obtained from a single preparation. When a molar concentration at 50% relaxation was determined, the response to chlorpromazine or amitriptyline was calculated as a percentage of the maximum relaxation obtained. The molar concentration at 50% relaxation was obtained visually from a plot of percent relaxation vs. log concentration of chlorpromazine or amitriptyline.

Human Platelet Aggregation Studies

Venous blood was taken from 5 healthy adults, who had not been treated with aspirin or indomethacin for the past 3 weeks. A mixture of 9 parts human blood to 1 part 3.8% sodium citrate was centrifuged at 200 G for 10 min and platelet-rich plasma (PRP) was removed, and the remainder was processed for platelet-poor plasma (PPP) by centrifuging at 2000 G for 10 min. The aggregation of platelet was induced by 1.0 U.M ADP (Sigma Chemical Co., St. Louis, Mo., U.S.A.), which is the critical concentration for the biphasic response of human platelet aggregation.33

| TABLE 1 | Effect of Chlorpromazine or Amitriptyline on Canine Basilar Artery Contracted by $10^{-5}$ M PGF$_2\alpha$, $10^{-5}$ M PGE$_2$, 1 mg/dl of Hb, or 100 µl/ml of serum |
| Agents | Conc. (M) | Reduced tension (mg) of basilar artery contracted by | |
| Before | | PGF$_2\alpha$ (7) | PGE$_2$ (8) | Hb (8) | serum (7) |
| chlorpromazine | $1 \times 10^{-6}$ | 2054 ± 499 | 1587 ± 346 | 2132 ± 406 | 1775 ± 333 |
| | $3 \times 10^{-6}$ | 168 ± 82 | 276 ± 103 | 285 ± 108 | 217 ± 115 |
| | $1 \times 10^{-5}$ | 899 ± 193 | 748 ± 132 | 799 ± 252 | 693 ± 130 |
| | $3 \times 10^{-5}$ | 1441 ± 281 | 1128 ± 246 | 1416 ± 320 | 1196 ± 207 |
| | $1 \times 10^{-4}$ | 1782 ± 362 | 1323 ± 286 | 1665 ± 393 | 1290 ± 302 |
| | $3 \times 10^{-4}$ | 2021 ± 418 | 1622 ± 336 | 2033 ± 458 | 1620 ± 314 |
| Papaverine | $1 \times 10^{-6}$ | 2471 ± 606 | 1890 ± 444 | 2497 ± 442 | 2132 ± 384 |
| molar conc. at 50% relaxation (10^{-6} M) | 6.2 ± 1.5 | 5.9 ± 1.6 | 7.2 ± 1.4 | 7.7 ± 1.4 |
| Before | | 2035 ± 281 | 1663 ± 348 | 2100 ± 514 | 1937 ± 393 |
| amitriptyline | $1 \times 10^{-6}$ | 121 ± 47 | 118 ± 65 | 201 ± 165 | 119 ± 84 |
| | $3 \times 10^{-6}$ | 325 ± 147 | 347 ± 130 | 567 ± 243 | 341 ± 108 |
| | $1 \times 10^{-5}$ | 685 ± 239 | 637 ± 204 | 990 ± 420 | 715 ± 231 |
| | $3 \times 10^{-5}$ | 1176 ± 190 | 1084 ± 226 | 1296 ± 408 | 1258 ± 316 |
| | $1 \times 10^{-4}$ | 1602 ± 221 | 1522 ± 288 | 1656 ± 408 | 1785 ± 457 |
| Papaverine | $1 \times 10^{-6}$ | 2465 ± 298 | 2003 ± 439 | 2487 ± 480 | 2291 ± 411 |
| molar conc. at 50% relaxation (10^{-6} M) | 3.9 ± 1.6 | 2.8 ± 1.2 | 3.5 ± 2.3 | 3.1 ± 1.8 |

Conc. = concentration of chlorpromazine, amitriptyline, or papaverine. 
A molar concentration at 50% relaxation of chlorpromazine or amitriptyline is calculated on the assumption that the maximum relaxation induced with $1 \times 10^{-4}$ M papaverine is taken as 100%. Number in parenthesis indicates number of basilar artery used.
ADP in \( n \)m
\( 1x10^{-6} \) and \( 3x10^{-5} \) M for PGF\(_2\alpha\)-induced contraction, \( 5.9 \pm 1.6 \times 10^{-6} \) and \( 2.8 \pm 1.2 \times 10^{-5} \) M for PGE\(_2\)-induced contraction, \( 7.2 \pm 1.4 \times 10^{-6} \) and \( 3.5 \pm 2.3 \times 10^{-5} \) M for Hb-induced contraction, and \( 7.7 \pm 1.4 \times 10^{-6} \) and \( 3.1 \pm 1.8 \times 10^{-5} \) M for serum-induced contraction, respectively. Amitriptyline was much less potent in relaxing the canine basilar artery contraction than chlorpromazine.

Human Platelet Aggregation Studies

The platelet aggregation in response to 1.0 \( \mu \)M ADP was variable from person to person. A typical tracing of ADP-induced platelet aggregation when chlorpromazine or amitriptyline was given, is shown in figure 1. Chlorpromazine and amitriptyline were effective in a dose-dependent manner for the inhibition of platelet aggregation induced by 1.0 \( \mu \)M ADP, as exhibited in table 2, in which the aggregation rates at 1 and 5 min after the addition of ADP are shown if the transmission of PPP is taken as 100% and percent inhibitions are calculated from the aggregation rates induced by ADP alone and by chlorpromazine or amitriptyline plus ADP. If the platelet aggregations at 1 and 5 min after the addition of ADP indicated the primary and the secondary aggregations of platelet, respectively, the inhibition of the primary aggregation by chlorpromazine or amitriptyline was weaker than that of the secondary, and more amount of chlorpromazine or amitriptyline was needed to inhibit the primary aggregation. The molar concentrations at 50% inhibition of secondary aggregation by chlorpromazine and amitriptyline were \( (5.7 \pm 2.3) \times 10^{-5} \) M and \( (11.1 \pm 5.1) \times 10^{-5} \) M, respectively, as shown in table 2. Amitriptyline was less potent in inhibiting human platelet aggregation than chlorpromazine.

Discussion

Blaumanis and Grady\(^{34}\) reported that a topical application of \( 10^{-7} \) to \( 10^{-4} \) M chlorpromazine led to a prompt relaxation of the spastic cerebral artery segment induced by electrical, mechanical, or chemical stimuli or by subarachnoid hemorrhage. The sustained contraction of the canine basilar artery induced by PGF\(_2\alpha\), PGE\(_2\), Hb, or serum in the present study was relaxed in a dose-dependent manner by chlorpromazine or amitriptyline. Alpha-adrenergic, serotonergic and histaminergic \( H_1 \) mechanisms were not involved in the contractile responses of the canine basilar artery to PGF\(_{2\alpha}\) and PGE\(_2\).\(^{25}\) In addition, alpha-adrenergic, histaminergic \( H_1 \) and angiotensin-related mechanisms were not involved in the contractile responses to Hb and serum.\(^{26}\) Finally, the relaxation induced by chlor-
promazine or amitriptyline was not affected by treatment with adrenergic or cholinergic blocking agents such as propranolol and atropine, suggesting that chlorpromazine or amitriptyline does not work through various receptors described above.

Recent studies of smooth muscle cells have suggested that contraction is regulated by a light chain kinase, which is activated by calmodulin in the presence of Ca\(^{2+}\) and that activation of the kinase enhances the activity of actomyosin ATPase and the contraction of the myosin system.\(^{10,12}\) Hidaka et al.\(^{16}\) reported that naphthalenesulfonamide derivatives or psychotropic drugs such as chlorpromazine and amitriptyline induced relaxed relaxation of rabbit aortic strips contracted by various agonists such as KCl, CaCl\(_2\), norepinephrine, histamine and PGF\(_{2\alpha}\) and inhibited the superprecipitation of aorta smooth muscle actomyosin by addition of ATP. The psychotropic drugs such as chlorpromazine and amitriptyline showed a high-affinity, Ca\(^{2+}\)-specific binding to calmodulin, forming a calmodulin-calcium-psychotropic complex which cannot activate the calmodulin-sensitive form of enzymes.\(^{17-22}\)

A Scatchard analysis of the binding of chlorpromazine to calmodulin revealed two sets of binding sites; one set of high-affinity sites (K\(_d\) = 5 \(\mu\)M, N = 3 sites per molecule) and the second set of low-affinity site (K\(_d\) = 130 \(\mu\)M, N = about 17 sites per molecule), and the high-affinity binding was dependent on the presence of calcium and the low-affinity sites were calcium independent.\(^{19}\) The degree to which psychotropic drugs bind to calmodulin is directly related to their ability to inhibit the activation of phosphodiesterase.\(^{19}\) \(I_{50}\) values for the inhibition of activated phosphodiesterase by chlorpromazine and amitriptyline were 42 and 3\(\times\)10\(^{-5}\) \(\mu\)M, respectively.\(^{17,21}\) \(I_{50}\) value for phosphodiesterase inhibition by trifluoperazine was about 10 times greater than K\(_d\) value for specific binding of trifluoperazine to calmodulin.\(^{18}\) The molar concentrations at 50% relaxation by chlorpromazine or amitriptyline were 5.9 to 7.7 \(\mu\)M or 28 to 39 \(\mu\)M for isolated canine basilar artery, and low as compared to the K\(_d\) value for phosphodiesterase by chlorpromazine and amitriptyline\(^{17,22}\) or to \(I_{50}\) values of chlorpromazine and amitriptyline for the relaxation of rabbit aorta contracted by 0.5 \(\times\)10\(^{-6}\) M PGF\(_{2\alpha}\),\(^{35}\) possibly due to the difference of species or to the characteristic of cerebral artery.

Hogaboom and Fedan\(^{36}\) reported that calmodulin in the canine tracheal smooth muscle directly stimulated the activity of a microsomal Ca\(^{2+}\)-transport ATPase and that in addition to the initiation of contraction, Ca\(^{2+}\)-calmodulin interactions might regulate the rate and degree of smooth muscle relaxation by stimulating the removal of Ca\(^{2+}\) from the myoplasm and facilitating the dissociation of Ca\(^{2+}\) from the contractile proteins. However, if chlorpromazine or amitriptyline inhibits the transmembrane Ca\(^{2+}\)-influx, it is likely that the relaxing action of these drugs is dependent on the extracellular calcium concentration and that this relaxation would be abolished by extracellular calcium in excess amount. Kanamori et al.\(^{37}\) showed that 1 \(\times\)10\(^{-6}\) and 3 \(\times\)10\(^{-6}\) M chlorpromazine shifted the dose-response curve of rabbit aortic strips for CaCl\(_2\) to the right, indicating that Ca\(^{2+}\)-induced contraction was inhibited in a competitive fashion. Since the effect of chlorpromazine on phospholipid, Ca\(^{2+}\)-dependent protein kinase was partially responsible for its pharmacological actions,\(^{38}\) chlorpromazine would be an effective Ca\(^{2+}\)-influx inhibitor. The pharmacological action of amitriptyline for Ca\(^{2+}\)-flux is unknown, but chlorpromazine might induce the relaxation of the canine basilar artery by acting as calmodulin antagonist and Ca\(^{2+}\)-flux inhibitor.

The inhibitory effects of chlorpromazine and amitriptyline on platelets have already been well studied.\(^{39-42}\) Irreversible ADP-induced platelet aggregation shifted promptly from primary to secondary aggregation, and platelet release reaction initiated simultaneously with the beginning of the secondary aggregation.\(^{43}\) The secondary aggregation of platelet was inhibited in a dose-dependent manner by the addition of chlorpromazine or amitriptyline in the present study. Serotonin uptake by platelets was inhibited by chlorpromazine,\(^{40}\) and single phase reversible aggregation triggered by serotonin in PRP was prevented by trifluoperazine.\(^{44}\) However, serotonin uptake was also inhibited by ouabain,\(^{45}\) which did not prevent secretion-associated aggregation,\(^{46}\) and, in general, the abil-

### Table 2: Effect of Chlorpromazine or Amitriptyline on Human Platelet Aggregation Induced by 1.0 \(\mu\)M ADP

<table>
<thead>
<tr>
<th>Agents</th>
<th>Conc. (M)</th>
<th>1 min (%)</th>
<th>Aggregation rate % inhibition</th>
<th>5 min (%)</th>
<th>% inhibition</th>
</tr>
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<tbody>
<tr>
<td>Chlorpromazine</td>
<td>control</td>
<td>60.4 ± 10.7</td>
<td>73.8 ± 7.9</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>3 (\times)10(^{-6})</td>
<td>58.2 ± 8.7</td>
<td>71.2 ± 6.9</td>
<td>9</td>
<td>63.6 ± 15.5</td>
</tr>
<tr>
<td></td>
<td>1 (\times)10(^{-5})</td>
<td>55.0 ± 10.4</td>
<td>71.2 ± 6.9</td>
<td>9</td>
<td>63.6 ± 15.5</td>
</tr>
<tr>
<td></td>
<td>3 (\times)10(^{-5})</td>
<td>57.6 ± 10.4</td>
<td>58.4 ± 16.4</td>
<td>5</td>
<td>78.4 ± 19.2</td>
</tr>
<tr>
<td></td>
<td>1 (\times)10(^{-4})</td>
<td>49.2 ± 12.5</td>
<td>15.6 ± 13.0</td>
<td>19</td>
<td>15.6 ± 13.0</td>
</tr>
<tr>
<td></td>
<td>3 (\times)10(^{-4})</td>
<td>49.8 ± 7.9</td>
<td>72.6 ± 11.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>control</td>
<td>49.8 ± 7.9</td>
<td>72.6 ± 11.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 (\times)10(^{-5})</td>
<td>45.4 ± 7.9</td>
<td>69.0 ± 13.6</td>
<td>9</td>
<td>69.0 ± 13.6</td>
</tr>
<tr>
<td></td>
<td>3 (\times)10(^{-5})</td>
<td>44.6 ± 9.0</td>
<td>67.8 ± 16.0</td>
<td>10</td>
<td>67.8 ± 16.0</td>
</tr>
<tr>
<td></td>
<td>1 (\times)10(^{-4})</td>
<td>39.8 ± 7.9</td>
<td>38.6 ± 21.4</td>
<td>20</td>
<td>38.6 ± 21.4</td>
</tr>
<tr>
<td></td>
<td>3 (\times)10(^{-4})</td>
<td>29.0 ± 8.1</td>
<td>5.4 ± 3.9</td>
<td>42</td>
<td>5.4 ± 3.9</td>
</tr>
</tbody>
</table>

Aggregation rates and their percent inhibitions at 1 and 5 min after the addition of ADP are shown.
ity of drugs to block serotonin uptake did not parallel their ability to block platelet aggregation. It is unlikely, therefore, that the inhibitory activity of chlorpromazine and possibly amitriptyline on platelets involves prevention of serotonin uptake. Chlorpromazine was bound preferentially to phosphatidylserine and phosphatidylinositol in the inner bilayer of the platelet membrane, and produced its inhibitory effect on the platelet function in part by changing the organization of the membrane bilayer.

Platelet myosin light chain kinase has been identified as a Ca^{2+}-dependent enzyme that requires calmodulin for its activity, and a calmodulin-mediated system, such as Ca^{2+}-dependent phosphorylation plays an important role in the release reaction of platelet. Calmodulin antagonists such as trifluoperazine and naphthalenesulfonamide derivatives inhibited secondary aggregation and release reaction of platelet. Grinstein and Furuya showed approximately 4100 calmodulin binding sites per platelet. Half-maximal inhibition for secondary aggregation of platelet was attained with 57 μM chlorpromazine or 111 μM amitriptyline in the present study, which were similar, though not identical, to the reported affinity of calmodulin for these substances. The disagreement could reflect restricted diffusion of these drugs into the compartment where calmodulin is located, but could also be an indication of an unspecific mode of inhibition.

In addition, chlorpromazine affected ATP-dependent calcium transport by a micromolar fraction from human platelets. Platelet phospholipase A₂, which is involved in the release of arachidonic acid from certain phospholipids, has also been reported to be stimulated by calmodulin in membrane preparations, and inhibition of arachidonic acid mobilization by trifluoperazine has recently been reported. If chlorpromazine and amitriptyline have an inhibitory effect on arachidonic acid mobilization, similar to that by trifluoperazine, the decreased formation, of thromboxane A₂, might prevent platelet aggregation and contraction of cerebral artery. The pharmacological effects of chlorpromazine and amitriptyline on platelets may result from a combination of several types of molecular interactions and cannot be explained by a single mechanism.

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