Effects of ML-9 on experimental delayed cerebral vasospasm

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Experimental delayed cerebral vasospasm was produced in a two-hemorrhage canine model. The spastic basilar artery was exposed via the transclival route under a surgical microscope, and was dilated by the topical application of 1-(5-chloronaphthalenesulfonyl)-1H-hexa-hydro-1,4-diazepine (ML-9), a selective antagonist of myosin light chain kinase. Dilation was dose-dependent, with a median effective dose (± standard deviation) of 51.4 ± 6.9 μM. In addition, 50 μM of ML-9 was injected into the cisterna magna until the intracranial pressure (ICP) reached 200 mm H$_2$O for 30 minutes, including a complete reversal of angiographic delayed vasospasm in three of seven dogs; in contrast, 150 μM of ML-9 was infused at 1.52 ml/min into the vertebral artery for 30 minutes, producing little dilation of the spastic basilar artery. In another study, the intracisternal perfusion of 50 μM of ML-9 at 1.48 ml/min for 30 minutes in dogs with an ICP of less than 200 mm H$_2$O produced no serious electroencephalographic abnormalities, and the mean arterial blood pressure and pulse rate remained normal; no neurological deficits or significant histological abnormalities ascribable to the intracisternal ML-9 were found.

KEY WORDS  • cerebral vasospasm  • myosin light chain kinase  • ML-9  • subarachnoid hemorrhage  • dog

A recent report has suggested that cerebral vasospasm in the two-hemorrhage canine model of subarachnoid hemorrhage (SAH) does not reflect severe damage to the intracellular mechanism responsible for the contraction of the vascular smooth muscle of basilar artery as mediated by myosin light chain kinase.$^{17}$ A previous study$^{10}$ found that chlorpromazine, a calmodulin antagonist, was an effective reagent for reversing of experimental delayed cerebral vasospasm when applied topically, suggesting that chlorpromazine inhibits the Ca$^{2+}$-calmodulin-dependent activity of myosin light chain kinase by inactivating the calmodulin, which is an important functional protein in the contraction of smooth muscle.$^{5,14}$ However, calmodulin activates a variety of enzymes, thereby exerting a pleiotropic effect on various cellular functions: calmodulin antagonists, therefore, appear to represent an unsatisfactory means to isolate and elucidate the function of myosin light chain kinase among many target enzymes of calmodulin in delayed vasospasm. In this respect, 1-(5-chloronaphthalenesulfonyl)-1H-hexa-hydro-1,4-diazepine (ML-9) inhibits selectively the catalytic activity of smooth-muscle myosin light chain kinase by binding at the adenosine triphosphate (ATP)-binding site of the enzymes,$^{9,11}$ and is examined as a therapeutic agent for delayed vasospasm in a two-hemorrhage canine model.

Materials and Methods

Delayed Cerebral Vasospasm Model

Experimental delayed cerebral vasospasm was produced by two successive administrations, 2 days apart, of 5 ml fresh autogenous arterial blood into the cisterna magna of 16 adult mongrel dogs, 12 to 16 kg in weight, sedated with intramuscular ketamine hydrochloride (10 mg/kg) and then with intravenous pentobarbital sodium (15 mg/kg), as reported by Varsos, et al.$^{18}$ Transfemoral vertebral angiography was carried out before and 7 days after the first intracisternal injection of blood, and the diameter of the basilar artery was measured on magnified angiograms.

Study Groups

Experimental Protocol. The care of these animals met the United States Public Health Service standards. The animals were anesthetized with intravenous pentobarbital sodium (15 mg/kg), and maintained with a nitrous oxide-oxygen mixture (70%:30%) delivered by an intermittent positive-pressure ventilator in open cir-
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cuit.* Muscle relaxation was assured with an intravenous half-hourly injection of pancuronium bromide (0.08 mg/kg). Body temperature was kept close to 37°C with a heating blanket, and arterial blood pressure was continuously monitored with a strain-gauge transducer connected to a cannula in the femoral artery.† Arterial CO₂ tension was measured by a pH/blood gas monitor‡ and kept at a mean (± standard deviation) of 32 ± 3 torr throughout the experiment by adjusting the respiratory pump or by adding CO₂ to the inspired gas. When the occurrence of delayed cerebral vasospasm was angiographically confirmed 7 days after the first intracisternal injection of blood, the animals were separated into three groups. In one group the effects of treatment with topical ML-9 application were studied, in another the results of intracisternal ML-9 pumping, and in the third the effects of intravascular ML-9 infusion. Mean arterial blood pressure (MABP) and pulse rate were monitored during the treatment. The ML-9 was dissolved in dimethyl sulfoxide as a 10-mM stock solution and further diluted in saline. The final concentration of dimethyl sulfoxide used had no effect on the caliber of the normal and spastic basilar arteries.

**Topical Application.** Five dogs were included in the topical ML-9 application study. Under a surgical microscope, the spastic basilar artery was exposed by gently removing the clivus and by careful incision of the dura mater and arachnoid. Blood clot around the spastic basilar artery and its branches was meticulously removed without mechanical stimulation of the spastic basilar artery, and the operative site was briefly washed with warm saline. After the spastic basilar artery was photographed, dose-response data were obtained by topical application of ML-9 and increasing the concentrations by a factor of about three, while the previous dose remained in contact with the spastic basilar artery and showed a steady response. The mean diameter of each spastic basilar artery was calculated by measuring the true diameters at three predetermined levels of the spastic basilar artery. At the end of each experiment, 4 mM ethylene-glycol-bis(β-amino-ethylether)N,N′-tetra-acetic acid (EGTA) was added and, for purposes of analysis, the relaxation induced by EGTA was considered as 100%. The molar concentration at 50% relaxation (ED₅₀) was computed from percent relaxation versus log concentration of ML-9.

**Intracisternal Pumping.** Seven dogs were available for the intracisternal ML-9 pumping study. Direct intracisternal perfusion of ML-9 is not easy in this model of vasospasm, mainly because of the difficulty in puncturing the chiasmatic cistern due to the presence of massive blood clot. Instead, a No. 22 spinal needle was percutaneously inserted into the cisterna magna and connected to a manometer, in which an opening was made 200 mm above the level of the needle, as reported previously. The ML-9 solution (50 μM) was slowly injected into the cisterna magna until the intracranial pressure (ICP) reached 200 mm H₂O and was maintained at that level for 2 minutes. Cerebrospinal fluid was then slowly removed until the ICP was reduced to 0 mm H₂O. The intracisternal pumping of 50 μM ML-9 was repeated, with ICP increased to 200 mm H₂O and maintained at that level for 30 minutes. At that stage, transfemoral vertebral angiography was performed to measure the diameter of the spastic basilar artery.

**Intravascular Infusion.** Four dogs were included in the intravascular ML-9 study. The ML-9 solution (150 μM) was infused at a rate of 1.52 ml/min for 30 minutes into the vertebral artery with an infusion-withdrawal pump. Transfemoral vertebral angiography was carried out to examine the effect of ML-9 infusion on the diameter of the spastic basilar artery.

**Intracisternal ML-9 Infusion in Normal Dogs.** Six normal mongrel dogs, 11 to 15 kg in weight, were anesthetized with intravenous pentobarbital sodium (15 mg/kg). The technique for maintenance of body temperature and blood gas pressure was as described previously. Three screws were inserted into the midline of the frontal bone and both parietal bones, respectively, for electroencephalographic (EEG) recording. Two No. 22 spinal needles were inserted percutaneously into the chiasmatic cistern and the cisterna magna, respectively, to perfuse 50 μM of ML-9 at a rate of 1.48 ml/min for 30 minutes with the infusion-withdrawal pump. During perfusion, the loading fluid overflowed from the opening in the manometer to prevent increased ICP, as described previously.

Pulse rate, EEG recordings, and MABP were monitored during the perfusion. The EEG activity was amplified and filtered (0.5 to 100 Hz), recorded on a polygraph, and stored on magnetic tape. Data were analyzed offline. The sampling frequency was 100 Hz. Power was estimated with a signal processor for periods of 28 seconds by averaging seven 4-second short-time spectra obtained before ML-9 perfusion and at 5, 10, 15, 20, and 30 minutes after the beginning of the perfusion. Numerical values were obtained for delta (0 to 4 Hz), theta (4 to 8 Hz), alpha (8 to 12 Hz), and beta (12 to 20 Hz) bands. Data were expressed as the mean ± standard deviation of relative power and were statistically analyzed by a two-tailed t-test for uncorrelated pairs.

Normal animals were sacrificed 7 days after the intracisternal perfusion of ML-9. The brains were fixed...
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FIG. 1. Representative vertebral angiograms of a basilar artery before (a) and 7 days after (b) the first intracisternal injection of blood. The exposed spastic basilar artery (c) is dilated in a dose-dependent manner by topical applications of ML-9 at doses of $1 \times 10^{-6}$ M (d), $3 \times 10^{-6}$ M (e), $1 \times 10^{-5}$ M (f), $3 \times 10^{-5}$ M (g), $1 \times 10^{-4}$ M (h), and $3 \times 10^{-4}$ M (i), and by 4 mM EGTA (j).

by perfusion through the vertebral artery of 10% buffered formalin under a pressure of 120 to 140 torr. Brain sections were stained by hematoxylin and eosin and Klüver-Barrera method.

Results

Effects of Topical ML-9 Application

The diameters of the spastic basilar arteries were reduced to between 42% and 70% of control caliber 7 days after the first intracisternal injection of blood (Table 1). A representative vertebral angiogram of delayed cerebral vasospasm is shown in Fig. 1. A brief washing with warm saline after careful removal of the blood clot around the spastic basilar artery did not induce a significant change in the caliber of the spastic basilar artery. The spastic basilar arteries were dilated in a dose-dependent manner by a topical application of ML-9 at doses of $1 \times 10^{-6}$ to $3 \times 10^{-4}$ M (Table 1 and Fig. 1). Taking the relaxation induced by 4 mM EGTA as 100%, the ED$_{50}$ values for relaxation of spastic basilar artery by ML-9 were 43 to 62 µM, and their mean ± standard deviation value was 51.4 ± 6.9 µM (Table 1). No significant changes were found in MAP or pulse rate during the topical application of ML-9.

Effect of Intracisternal ML-9 Pumping

In two of seven animals with delayed cerebral vasospasm (46.4% and 65.3% of control caliber), there was no angiographic change in arterial diameter after the intracisternal pumping of 50 µM of ML-9 for 30 minutes (Dogs 12 and 8, respectively, Table 2). A massive blood clot was found around the basilar artery in both dogs at autopsy (Fig. 2). In Dogs 7 and 11 (with vasospasm of 55.6% and 43.9% of control caliber, respectively), delayed cerebral vasospasm was moderately reversed after the intracisternal pumping of ML-9, demonstrating a moderate amount of blood clot around the basilar artery. The angiographic delayed cerebral vasospasm in Dogs 6, 9, and 10 (66.2%, 53.7%, and 61.5%...
Experimental delayed cerebral vasospasm

**TABLE 1**
Response of exposed basilar artery to topical application of ML-9 and EGTA*

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Spastic Artery Diameter (mm)</th>
<th>% Change</th>
<th>Response (mm) to Various ML-9 Doses:</th>
<th>Response (mm) to 4 mM EGTA</th>
<th>ED50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.72</td>
<td>70%</td>
<td>1 x 10^-6 M 3 x 10^-6 M 1 x 10^-5 M 3 x 10^-5 M 1 x 10^-4 M 3 x 10^-4 M</td>
<td>1.49</td>
<td>43</td>
</tr>
<tr>
<td>2</td>
<td>0.75</td>
<td>64%</td>
<td>1 x 10^-6 M 3 x 10^-6 M 1 x 10^-5 M 3 x 10^-5 M 1 x 10^-4 M 3 x 10^-4 M</td>
<td>1.21</td>
<td>62</td>
</tr>
<tr>
<td>3</td>
<td>0.79</td>
<td>42%</td>
<td>1 x 10^-6 M 3 x 10^-6 M 1 x 10^-5 M 3 x 10^-5 M 1 x 10^-4 M 3 x 10^-4 M</td>
<td>1.09</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>0.89</td>
<td>65%</td>
<td>1 x 10^-6 M 3 x 10^-6 M 1 x 10^-5 M 3 x 10^-5 M 1 x 10^-4 M 3 x 10^-4 M</td>
<td>1.01</td>
<td>45</td>
</tr>
<tr>
<td>5</td>
<td>0.93</td>
<td>63%</td>
<td>1 x 10^-6 M 3 x 10^-6 M 1 x 10^-5 M 3 x 10^-5 M 1 x 10^-4 M 3 x 10^-4 M</td>
<td>1.24</td>
<td>55</td>
</tr>
</tbody>
</table>

*ED50 indicates molar concentration at 50% relaxation of the spastic basilar artery when the relaxation induced by 4 mM EGTA is taken as 100%. Mean (± standard deviation) ED50 for all five dogs: 51.4 ± 6.9 µM. EGTA = ethylene-glycol-bis-(β-amino-ethyl)ether-N,N'-tetra-acetic acid.

**Effect of Intravascular ML-9 Infusion**

As a preliminary experiment, 50 µM and 100 µM of ML-9 were infused into the vertebral artery for 30 minutes to examine the effect on the angiographic delayed cerebral vasospasm, but no marked change was observed.

of control caliber, respectively) was largely reversed after the intracisternal pumping of ML-9, showing a relatively small amount of blood clot around the basilar artery (Fig. 2). There was no evidence of significant changes in MABP or pulse rate during the intracisternal pumping of ML-9.
found in the diameter of the spastic basilar artery. On the other hand, a bolus ML-9 injection of 500 μM into the vertebral artery induced temporary severe hypotension. Consequently, a 150-μM dose of ML-9 was used in the intravascular infusion study. The diameters of the spastic basilar arteries after intravascular infusion of a 150-μM dose of ML-9 for 30 minutes are shown in Table 3. There was no evidence of significant dilation of spastic basilar artery. No significant changes were found in MABP or pulse rate during the intravascular infusion of ML-9.

Effect of Intracisternal ML-9 on Normal Animals

Quantitative analysis of the power spectra showed that the relative powers of delta, theta, alpha, and beta waves on EEG recording were 44.0% ± 11.2%, 26.0% ± 6.7%, 19.8% ± 5.0%, and 10.2% ± 3.2%, respectively, before the intracisternal perfusion of 50 μM of ML-9, and did not change significantly during the intracisternal perfusion of ML-9 (Table 2). Mean ± standard deviation values of MABP and pulse rate were 112.9 ± 9.1 mm Hg and 146.8 ± 29.4 beats/min, respectively, before the intracisternal perfusion of 50 μM of ML-9, and did not change significantly during the intracisternal perfusion of ML-9. In addition, no neurological deficits due to the intracisternal perfusion of ML-9 were found. No significant histological changes ascribable to ML-9 were found in either the neurons or the glial cells.

Discussion

Although the regulation of actin-myosin interaction in smooth muscle by Ca++ is not fully understood, it has been generally accepted that the Ca++-dependent phosphorylation of the myosin light chain is necessary for vascular contraction. Myosin light chain kinase is a Ca++-calmodulin-dependent enzyme which catalyzes the transfer of γ-phosphate from ATP to the regulatory light chain of smooth-muscle myosin. This reaction, which is obligatory for the stimulation of myosin adenosine triphosphatase in smooth muscle, is a prerequisite for tension development. It has been found that ML-9 is a potent selective inhibitor of myosin light chain kinase both dependent and independent of Ca++-calmodulin, with a similar concentration dependence; however, it is less potent in suppression of other calmodulin-dependent enzymes, protein kinase C, and cyclic adenosine monophosphate-dependent protein kinase.

In addition, ML-9 is a competitive inhibitor with respect to ATP and a noncompetitive inhibitor with respect to the phosphate acceptor. Thus, ML-9 is a new type of vascular relaxant, producing a dose-dependent relaxation of rabbit mesenteric arterial strips contracted by higher K+, CaCl2, norepinephrine, serotonin, histamine, or angiotensin II; the concentrations of ML-9 producing a 50% inhibition of sustained contraction (the IC50 values) were: 17.5 ± 0.4 μM for higher K+, 27.5 ± 7.9 μM for CaCl2, 28.2 ± 7.9 μM for norepinephrine, 19.5 ± 3.6 μM for serotonin, 87.1 ± 6.6 μM for histamine, and 11.0 ± 4.1 μM for angiotensin II. The mean ED50 value for the relaxation of delayed vasospasm by the topical application of ML-9 in the present study was 51.4 ± 6.9 μM. As ML-9 competes with ATP for several protein kinases, higher concentrations would be required to inhibit myosin light chain kinase as well as other kinases in vivo, because cells or tissues contain a larger amount of ATP.

The relaxation induced by ML-9 was not affected by treatment with adrenergic and cholinergic blocking agents, and ML-9 antagonized nonspecifically the contraction induced by various contractile agonists, such as CaCl2, norepinephrine, serotonin, histamine, and angiotensin II. Thus, ML-9-induced relaxation is not due to a block of membrane receptor-associated mechanisms, but ML-9 has an effect on more basic and common events in smooth-muscle contraction. In addition, ML-9 inhibited the Ca++-induced contraction in chemically skinned smooth-muscle cells of rabbit mesenteric artery, suggesting that ML-9 is not a calcium channel blocker.

Suzuki, et al., found no difference in the myosin light chain kinase activity of canine basilar artery between a control study and the two-hemorrhage model of SAH at any time for up to 10 days after the intracisternal injection of blood. However, myosin light chain phosphorylation was rapid and attained a maximum

### Table 2

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Vasosperm (% Control) Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>66.2</td>
<td>98.1</td>
</tr>
<tr>
<td>7</td>
<td>55.6</td>
<td>87.6</td>
</tr>
<tr>
<td>8</td>
<td>65.3</td>
<td>65.8</td>
</tr>
<tr>
<td>9</td>
<td>53.7</td>
<td>91.7</td>
</tr>
<tr>
<td>10</td>
<td>61.5</td>
<td>96.7</td>
</tr>
<tr>
<td>11</td>
<td>43.9</td>
<td>70.9</td>
</tr>
<tr>
<td>12</td>
<td>46.4</td>
<td>48.6</td>
</tr>
</tbody>
</table>

* An ML-9 dose of 50 μM was administered over 30 minutes.

### Table 3

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Vasosperm (% Control) Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>52.1</td>
<td>53.6</td>
</tr>
<tr>
<td>14</td>
<td>47.7</td>
<td>51.3</td>
</tr>
<tr>
<td>15</td>
<td>50.3</td>
<td>56.5</td>
</tr>
<tr>
<td>16</td>
<td>51.4</td>
<td>54.9</td>
</tr>
</tbody>
</table>

* An ML-9 dose of 150 μM was administered over 30 minutes.
level at 20 seconds in the early phase during a KC+-induced contraction of rabbit mesenteric artery; it then declined to near the basal level during the development of sustained contraction.\textsuperscript{2,3} In addition, the time course of the concentration of ML-9 sufficient for the reversal of delayed vasospasm is associated with the time course of vasospasm development; however, it does indicate that there has been no pathological alteration in myosin light chain kinase activity level in situ during vasospasm. Consequently, a large amount of intravascular ML-9, a prolonged intravascular infusion of ML-9, or both may be necessary for the reversal of delayed vasospasm. The present study suggests that delayed vasospasm may be reversed by the selective inhibition of myosin light chain kinase in the basilar artery and that the concentration of ML-9 sufficient for the reversal of delayed vasospasm is attainable by intracisternal perfusion without any serious effect on blood pressure or pulse rate. In addition, no neurological deficits, no serious EEG changes, and no significant histological abnormalities were found due to the intracisternal perfusion of ML-9. The various responses to intracisternal pumping of ML-9 of dogs with delayed vasospasm in the present study may be due to the technical difficulty of ML-9 perfusion into the chiasmatic cistern and to the amount of blood clot around the spastic basilar artery which prevents contact of ML-9 with the vessel wall. If the blood clot around the major cerebral arteries is carefully removed in early surgery, intracisternal perfusion of ML-9 might be successful for the treatment of delayed vasospasm. The concentration of ML-9 infused into the vertebral artery was three times higher than that administered into the cisterna magna, but there was no angiographic evidence of amelioration of delayed vasospasm by intravascular ML-9. It is probable that the concentration of ML-9 sufficient for the reversal of delayed vasospasm is not reached in the vascular smooth muscle when introduced by the intravascular route, although Sasaki, et al.,\textsuperscript{13} reported a barrier disruption in the major cerebral arteries following experimental SAH. The administration time of intravascular ML-9 (30 minutes in the present study) may be too short to display the effect of intravascular ML-9 on delayed vasospasm. Consequently, a large amount of intravascular ML-9, a prolonged intravascular infusion of ML-9, or both may be necessary for the reversal of delayed vasospasm. However, severe hypotension may result from a higher concentration of intravascular ML-9 or a prolonged intravascular infusion of ML-9 (as suggested by the occurrence of serious hypotension induced by a bolus injection of 500 \textmu M ML-9 in the present study), because the vascular smooth muscles in all vessels may be relaxed by inactivating vascular myosin light chain kinase.

References


\begin{table}
\centering
\caption{Effect on EEG data, MABP, and PR before and after intracisternal ML-9 perfusion*}
\begin{tabular}{|l|l|l|l|l|l|}
\hline
Parameter & Before & & & & \\
\hline
& Parameter & ML-9 perfusion & 5 min & 10 min & 15 min & 20 min & 30 min \\
\hline
EEG tracings & delta wave (%) & 44.0 ± 11.2 & 44.9 ± 11.7 & 40.1 ± 10.9 & 43.8 ± 9.7 & 45.6 ± 11.6 & 47.3 ± 9.4 \\
theta wave (%) & 26.0 ± 6.7 & 26.0 ± 6.3 & 27.4 ± 5.9 & 25.9 ± 6.5 & 23.6 ± 5.2 & 24.0 ± 5.3 \\
alpha wave (%) & 19.8 ± 5.0 & 18.8 ± 4.8 & 21.1 ± 4.9 & 19.9 ± 5.7 & 19.2 ± 5.2 & 19.1 ± 5.5 \\
beta wave (%) & 10.2 ± 3.2 & 10.3 ± 4.5 & 11.4 ± 3.5 & 10.4 ± 4.7 & 11.3 ± 4.8 & 9.6 ± 2.9 \\
MABP (mm Hg) & 112.9 ± 9.1 & 128.7 ± 13.1 & 132.7 ± 19.4 & 134.6 ± 19.1 & 136.0 ± 18.1 & 135.6 ± 18.2 \\
PR (beats/min) & 146.8 ± 29.4 & 165.5 ± 15.0 & 161.8 ± 20.5 & 159.2 ± 24.2 & 153.4 ± 27.1 & 145.4 ± 25.4 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{*} An ML-9 dose of 50 \textmu M was administered over 30 minutes. EEG = electroencephalographic; MABP = mean arterial blood pressure; PR = pulse rate.
the vascular contraction via the inhibition of myosin light chain phosphorylation. Mol Pharmacol 33:598–603, 1988


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