Activation of the Protein Kinase C–Mediated Contractile System in Canine Basilar Artery Undergoing Chronic Vasospasm

Tohru Matsui, MD; Makoto Sugawa, MSc; Hiroo Johshita, MD; Yoh Takuwa, MD; and Takao Asano, MD

We previously suggested that activation of the protein kinase C–mediated contractile system may participate in the occurrence of chronic cerebral vasospasm. In the present study, we compared segments of normal beagle basilar arteries in vitro with segments of arteries undergoing chronic vasospasm to determine the responsiveness to various agonists such as serotonin, prostaglandin F$_{2\alpha}$, and phorbol 12,13-diacetate as well as to external Ca$^{2+}$. We also compared the effects of W-7 (a calmodulin inhibitor), nicardipine (a calcium channel blocker), and H-7 (a protein kinase C inhibitor) on the spontaneous tonus of arterial segments stabilized at a resting tension of 3 g. Compared with normal segments, the responsiveness to each agonist in segments undergoing vasospasm was essentially unchanged whereas the responsiveness to external Ca$^{2+}$ was significantly decreased ($p<0.001$). In segments undergoing vasospasm the decrease in resting tension induced by W-7 was markedly diminished ($p<0.01$), that induced by nicardipine was unchanged, and that induced by H-7 was significantly increased ($p<0.01$). Our results indicate that spontaneous tonus due to activation of the protein kinase C system is significantly augmented in segments undergoing vasospasm. Thus this system, rather than the Ca$^{2+}$/calmodulin system, appears to play a major role in the occurrence of chronic vasospasm.


Past studies using various animal models of subarachnoid hemorrhage (SAH) have shown that cerebral arteries undergoing chronic vasospasm have diminished responsiveness to various agonists, decreased distensibility, and increased active tonus. Additionally, organic changes occur in the arterial wall and arterial narrowing is resistant to the application of various vasodilators, even Ca$^{2+}$ antagonists.

According to the widely accepted concept that contraction of arterial smooth muscle is dependent on the Ca$^{2+}$/calmodulin system, it has been conjectured that changes in active tonus cannot form a basis for severe chronic vasospasm. In agreement with Rasmussen et al, however, we have shown that phorbol 12,13-diacetate (PDA) induces potent contraction of the beagle basilar artery in either normal or Ca$^{2+}$-free Krebs-Henseleit solution. We ascribed this contraction to activation of the protein kinase C system because it was inhibited by protein kinase C inhibitors such as H-7 and staurosporine and because it was accompanied by phosphorylation of proteins other than 20-kd myosin light chain. Using the beagle two-hemorrhage SAH model, we have further shown that the basilar artery narrowing due to chronic vasospasm was significantly reversed by the topical application of protein kinase C inhibitors and that there was a highly significant linear correlation between the severity of angiographic narrowing of the basilar artery and the increase in arterial levels of an intrinsic protein kinase C activator throughout the evolution of chronic vasospasm. Thus, our preceding results suggest that active smooth muscle contraction due to protein kinase C activation is involved in the occurrence of chronic vasospasm. In this study, we examined whether the in vitro contractile activities of arterial segments excised from the basilar artery during chronic vasospasm would show enhanced activity of the protein kinase C system in response to various agonists.

Materials and Methods
We used 36 beagles, 12, eight, four, and 12 in the first, second, third, and fourth experiments, respec-

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Received December 20, 1990; accepted May 21, 1991.
tively. Eight of 12 dogs in the first and fourth experiments and four of eight dogs in the second experiment were subjected to two-hemorrhage SAH.

The SAH was induced as previously reported. Under barbiturate anesthesia, cerebral angiography via the vertebral artery was carried out just prior to the induction of SAH on day 0 (control). On day 7, angiography was repeated and the dog was killed by exsanguination. The basilar artery was excised and immediately immersed in Krebs-Henseleit solution at room temperature. Arteries obtained from normal beagles and those exposed to two-hemorrhage SAH were dissected under an operating microscope and cut into six ring segments 5 mm long. Each segment was mounted on rigid prongs inside a chamber, which was then filled with 5 ml of Krebs-Henseleit solution (NaCl 120, KCl 4.5, CaCl$_2$ 2.5, MgCl$_2$ 1.0, NaHCO$_3$ 27, KH$_2$PO$_4$ 1.0, and glucose 10 in millimolar concentrations; pH 7.4±0.05). The Krebs-Henseleit solution was then replaced with a Ca$^{2+}$-free Krebs-Henseleit solution containing 0.5 mM ethylene dinitetraacetic acid. After stabilization of the arterial segments in the chamber at a resting tension calculated from each dose-response curve. The Krebs-Henseleit solution was then aerated with a 5% CO$_2$/95% O$_2$ gas mixture and kept at 37°C. The segments were allowed to equilibrate for 120 minutes before the start of each experiment while increasing stretch was applied to stabilize at a basal tension of 3 g. Changes in isometric tension following drug application were monitored by transducers (Nihon Kohden TB611, Japan) connected to a six-channel polygraph recorder (Nihon Kohden MC 6000).

In the first experiment, responses to serotonin, prostaglandin F$_{2a}$ (PGF$_{2a}$), and PDA were compared between arterial segments from normal beagles (normal segments) and arterial segments from beagles exposed to two-hemorrhage SAH (spasm segments). Each test agent was added to the chamber in a cumulative fashion and the subsequent changes in resting tension were recorded. The half-maximum contraction (ED$_{50}$) was calculated from each dose–response curve. The Krebs-Henseleit solution was then replaced with a Ca$^{2+}$-free Krebs-Henseleit solution containing 0.5 mM ethylene diaminetetraacetic acid. After stabilization of the arterial segments in the chamber at a resting tension of 3 g, the responses to PDA were compared between the groups. The solution was again replaced with a Ca$^{2+}$-free, high-K$^+$ solution (NaCl 24.5, KCl 100, MgCl$_2$ 1.0, NaHCO$_3$ 27, KH$_2$PO$_4$ 1.0, and glucose 10, in millimolar concentrations; pH 7.4±0.05), and the contractile response to increasing concentrations of CaCl$_2$ was examined at a basal tension of 3 g.

In the second experiment, PDA was added to the chamber at submaximal concentrations (10$^{-6}$ M for the normal segments and 3×10$^{-6}$ M for the spasm segments). After the contractile response reached a plateau (approximately 45 minutes after the addition of PDA), 10$^{-6}$ M methysergide, 10$^{-6}$ M atropine, 3×10$^{-6}$ M diphenhydramine, 10$^{-6}$ M phentolamine, 10$^{-6}$ M R24571, 10$^{-7}$ M W-7 (a calmodulin inhibitor), 10$^{-4}$ M nicardipine, and 10$^{-3}$ and 10$^{-4}$ M H-7 were added in a cumulative fashion, and the subsequent changes in tension were recorded.

In the third experiment, normal segments were stretched to stabilize at resting tensions of 1, 2, 3, or 4 g in chambers containing aerated Krebs-Henseleit solution. At each resting tension 10$^{-6}$ M nicardipine, 10$^{-3}$ M H-7, and 10$^{-4}$ M papaverine were added to the chamber in a cumulative fashion and the subsequent changes in resting tension were recorded.

In the fourth experiment, normal and spasm segments were stretched and stabilized at a resting tension of 3 g in aerated Krebs-Henseleit solution. The decrease in tension following the cumulative additions of 10$^{-3}$ M W-7, 10$^{-6}$ M nicardipine, and 10$^{-3}$ and 10$^{-4}$ M H-7 were compared between the groups.

In the first and fourth experiments, the spasm segments were further divided into moderate spasm segments (basilar artery diameter >60% of control) and severe spasm segments (basilar artery diameter <60% of control) according to the results of angiography carried out on day 7, prior to arterial excision. In the second experiment, only normal and severe spasm segments were used.

Values are expressed as mean±standard deviation. Dunnett’s method or the Mann-Whitney $U$ test was used for statistical evaluation. Probability values less than 0.05 were considered to be significant.

Serotonin, diphenhydramine hydrochloride, atropine sulfate, R24571, PDA, and PGF$_{2a}$ were pur-

<table>
<thead>
<tr>
<th>Agent</th>
<th>Normal</th>
<th>Moderate spasm</th>
<th>Severe spasm</th>
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<tbody>
<tr>
<td></td>
<td>Tension</td>
<td>Concentration</td>
<td>Tension</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(M)</td>
<td></td>
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<tr>
<td>Serotonin</td>
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<td>Phorbol 12,13-diace tate</td>
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<td>Ca$^{2+}$</td>
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<td>3×10$^{-4}$</td>
<td>2.47±0.68</td>
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<tr>
<td>Ca$^{2+}$</td>
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<td>3×10$^{-4}$</td>
<td>2.56±0.41</td>
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<tr>
<td>CaCl$_2$</td>
<td>2.36±0.01</td>
<td>3×10$^{-5}$</td>
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*Ca$^{2+}$, Krebs-Henseleit solution; Ca$^{2+}$, calcium-free Krebs-Henseleit solution. *$p<0.01$ different from normal by Dunnett’s method.
Table 2. ED_{50} Value for Various Agents in Canine Basilar Artery Segments

<table>
<thead>
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<th>Normal</th>
<th>Moderate spasm</th>
<th>Severe spasm</th>
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<td></td>
<td>n</td>
<td>ED_{50}</td>
<td>n</td>
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<tr>
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<td>Prostaglandin_{E2}</td>
<td>19</td>
<td>4.05±2.34x10^{-7}</td>
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<tr>
<td>Phorbol 12,13-diacetate</td>
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<td></td>
</tr>
<tr>
<td>Ca(+)</td>
<td>10</td>
<td>9.5±4.21x10^{-8}</td>
<td>22</td>
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<tr>
<td>Ca(−)</td>
<td>10</td>
<td>2.19±0.75x10^{-6}</td>
<td>22</td>
</tr>
<tr>
<td>CaCl_{2}</td>
<td>12</td>
<td>2.5±1.12x10^{-4}</td>
<td>18</td>
</tr>
</tbody>
</table>

Ca(+) Krebs-Henseleit solution; Ca(−), calcium-free Krebs-Henseleit solution.

Results

In the first experiment, diameters of the moderate and severe spasm segments were 68.9±7.8% and 46.3±4.8% of control, respectively. As shown in Table 1, the maximum tension (T_{max}) developed in response to serotonin or PDA was significantly less in the severe spasm segments than in the normal segments (p<0.01). The T_{max} developed in response to PGF₂α or CaCl₂ was slightly reduced in the severe spasm segments, although the difference was not significant. The ED_{50} values for each agent are shown in Table 2. Although the ED_{50} values for serotonin, PGF₂α, and PDA did not change significantly, that for CaCl₂ increased significantly in the moderate and severe spasm segments (p<0.001), with a shift of the dose-response curve to the right.

Among the various agents tested, only the protein kinase C inhibitor H-7 had a significant and dose-dependent inhibitory effect on the contraction elicited by PDA (Figure 1). The normal and spasm segments showed similar responses to H-7. None of the neurotransmitter antagonists or calmodulin inhibitors (R24571 and W-7) or the calcium channel blocker (nicardipine) significantly inhibited PDA-induced contraction.

Figure 2 shows the decreases in isometric tension following the cumulative applications of nicardipine and H-7 at different initial resting tensions. The final addition of papaverine induced no further decrease. The decrease in tension induced by nicardipine tended to increase as the initial tension increased, becoming maximal at a beginning tension of 3 g. H-7 also caused a significant decrease in tension, although the decrease was considerably less than that induced by nicardipine. An abrupt increase in the decrease in tension induced by H-7 was observed at a resting tension of 3 g.

Table 3 shows the decrease in tension following the cumulative applications of W-7, nicardipine, and H-7. Compared with the normal segments, severe spasm segments showed a marked diminution in the response to W-7 (p<0.01). The response to nicardipine was similar among the groups. A highly significant (p<0.01) increase in the decrease in tension induced by H-7 was revealed in both the moderate and severe spasm segments. No significant difference in the total reduction of tension after the addition of all three agents was revealed among the groups.
In agreement with preceding reports,\textsuperscript{13,14} our study showed that the $T_{\text{max}}$ values for serotonin, PGF$_{2\alpha}$, and PDA were reduced in severe spasm segments whereas the respective ED$_{50}$ values remained unchanged. Hence, the responsiveness to each agent appears to remain essentially intact even in segments with severe vasospasm. It is noteworthy that spasm segments showed a diminished responsiveness to external Ca$^{2+}$ ($p<0.001$). Later discussion will be directed to the possible underlying mechanism.

In a previous study,\textsuperscript{15,16} in both normal and spastic segments contraction induced by PDA was inhibited by H-7 but not by the remaining agents, such as various antagonists to neurotransmitters, W-7, R24571, and nicardipine. That in vivo study, using the same battery of agents that applied directly to canine basilar artery segments undergoing chronic vasospasm, provided results similar to ours. Such an analogy between the results of in vitro and in vivo experiments lends support to the view that chronic vasospasm represents sustained smooth muscle contraction due to activation of protein kinase C.

In our fourth experiment, arterial segments were stabilized at a resting tension of 3 g, which is ascertained to give optimal dilatory responses to pharmacological agents. The response to W-7 was markedly diminished in severe spasm segments ($p<0.01$), underscoring the report of Sakaki et al.\textsuperscript{17} These data could be taken as an indication that the Ca$^{2+}$/calmodulin system does not play a pivotal role. In this regard, it is intriguing to note that the spasm segments showed a highly significant ($p<0.01$) increase in the decrease in tension induced by H-7. These data clearly indicate that spontaneous tonus due to protein kinase C activation is augmented in segments undergoing chronic vasospasm.

Protein kinase C activation within the canine basilar artery undergoing vasospasm is also supported by biochemical data showing a significant increase in the arterial content of 1,2-diacylglycerol, an intrinsic activator of the enzyme.\textsuperscript{12} Recently, we have shown that membrane translocation of protein kinase C is also significantly increased in the canine basilar artery (unpublished data). Thus, the results of our present study may be interpreted as indicating that activation of the protein kinase C system leads to augmentation of spontaneous tonus within the cerebral arteries.

Based on the above findings, the diminished contractile response to external Ca$^{2+}$ in the spasm segments deserves an attempt at explanation. First, the diminished availability of calmodulin hinders myosin light chain kinase activation even if the function of
the calcium channel is unaffected. Second, activation of protein kinase C, leading to stimulation of plasma membrane Ca\(^{2+}\)-ATPase and Na\(^+\)/Ca\(^{2+}\) exchange, causes suppression of the calcium-signaling pathway through an increased extrusion of intracellular Ca\(^{2+}\).\(^{18}\) Whereas these events are considered to be causally related to the diminished responsiveness to Ca\(^{2+}\) in the spasm segments, recent studies show that protein kinase C activation augments the sensitivity of myofilaments to Ca\(^{2+}\).\(^{19,20}\) Further investigation may be required regarding the above issue.

In summary, our present study shows that compared with normal segments, the responsiveness of spasm segments to various agonists such as serotonin, PGF\(_{2\alpha}\) and FDA was essentially unchanged whereas that to external Ca\(^{2+}\) was decreased. In the spasm segments, on the other hand, the portion of spontaneous tonus attributable to protein kinase C activation was significantly augmented. The results of this study, together with the already reported in vivo data,\(^{12}\) indicate that activation of the protein kinase C system, rather than of the Ca\(^{2+}/\)calmodulin system, plays a major role in the occurrence of chronic vasospasm.

Acknowledgments

The authors wish to express their thanks to Mrs. Haruko Tanaka for her excellent technical assistance and to Dr. Tohru Koide for the helpful suggestions and discussion throughout the course of this work.

References

Activation of the protein kinase C-mediated contractile system in canine basilar artery undergoing chronic vasospasm.
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doi: 10.1161/01.STR.22.9.1183

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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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