Cerebrovascular Alterations in Protein Kinase C–Mediated Constriction in Stroke-Prone Rats

John S. Smeda, PhD; Shelley King, MSc

Background and Purpose—Cerebrovascular pressure-dependent constriction may involve the smooth muscle production of diacylglycerol, which could facilitate constriction by activating protein kinase C (PKC). A dysfunctional PKC system could promote the loss of pressure-dependent constriction. We attempted to determine whether the alterations in pressure-dependent constriction in the middle cerebral arteries (MCAs) observed in relation to stroke development in Wistar-Kyoto stroke-prone spontaneously hypertensive rats (SHRsp) were associated with defects in the ability of the arteries to constrict in response to PKC activation.

Methods—MCAs were sampled from SHRsp before and after stroke development and in stroke-resistant Wistar-Kyoto spontaneously hypertensive rats. A pressure myograph was used to test the ability of the arteries to constrict in response to a 100 mm Hg pressure step and subsequently to contract in response to phorbol 12,13-dibutyrate in the presence of nifedipine (3 μmol/L).

Results—Pressure-dependent constriction and constriction in response to phorbol dibutyrate in the MCAs were inhibited by PKC inhibitors (staurosporine [40 nmol/L], chelerythrine [12 μmol/L], bisindolylmaleimide [5 μmol/L]), declined with age before stroke development in SHRsp, and were absent after stroke. There was a significant relationship between pressure- and phorbol dibutyrate–induced constriction (r=0.815, P<0.05).

Conclusions—Phorbol esters interact with the same activation site as diacylglycerol to stimulate PKC. An inability to constrict in response to phorbol dibutyrate may reflect unresponsiveness to diacylglycerol and may contribute to the loss of pressure-dependent constriction associated with stroke in the MCAs of SHRsp. The loss of this autoregulatory function before stroke could increase the risk of cerebral hemorrhage. (Stroke. 1999;30:656-661.)

Key Words: cerebral arteries ■ phorbol 12,13-dibutyrate ■ protein kinase C ■ stroke ■ rats

Vascular constriction in response to elevations in blood pressure facilitates the autoregulation of blood flow.1 Elevations in blood pressure (which might increase blood flow) are counteracted by increased vascular resistance to flow, which serves to maintain vascular blood flow constant. The signal transduction mechanisms promoting cerebrovascular pressure-dependent constriction (PDC) are not fully understood. Studies of the cerebral and renal vasculatures from a variety of animal species have indicated that PDC requires the presence of extracellular Ca2+ and is inhibited by L-type Ca2+ channel antagonists.2–5 Protein kinase C (PKC) activation has also been implicated in the promotion of PDC. PKC inhibitors such as H-7, staurosporine, and calphostin C inhibit PDC in pressurized arteries.6–8 The application of the PKC activator indolactam allows PDC to occur in vascular segments that normally lack this function6 and enhances pressure-dependent tone in cerebral arteries.7,8 The synthesis of the above information has led to the development of hypotheses suggesting that an influx of Ca2+ through voltage-gated Ca2+ channels (perhaps in response to pressure-dependent smooth muscle depolarization)7,8 or through stretch-operated cation channels9 may act in conjunction with PKC activation to promote PDC.

Previous studies we performed have indicated that the ability of middle cerebral arteries (MCAs) to elicit constriction in response to pressure declines before stroke in Wistar-Kyoto stroke-prone spontaneously hypertensive rats (SHRsp) and is absent after stroke development.10 In the present study we attempted to determine whether PDC of MCAs was inhibited by PKC inhibitors, and we assessed the possibility that a defective PKC system may contribute to the loss of PDC in the MCAs of SHRsp in relation to stroke development.

Materials and Methods

Rats used in the study were taken from a colony housed in the animal facilities at the Memorial University Health Science Center (St John’s, Newfoundland, Canada). All experiments were done with institutional approval in a manner consistent with the guidelines of the Canadian Council on Animal Care. Unless specified otherwise, male rats were used in the study. The rats were fed a Japanese-style...
After PDC was measured, the arteries were maximally vasodilated with 3 μmol/L nifedipine. The above concentration of nifedipine is as effective in promoting maximal vasodilation as Ca\(^{2+}\)-free Krebs' saline containing 4 mmol/L EGTA. Therefore, differing levels of basal tone due to the presence of variable levels of PDC between the groups were abolished in the MCAs before the introduction of phosphodiesterase. The presence of nifedipine also eliminated the occurrence of constriction in response to PKC-activated \(\text{Ca}^{2+}\)-channel opening. Consequently, the possibility of differential constriction in response to phosphodiesterase occurring as a result of altered \(\text{Ca}^{2+}\)-channel function was eliminated. The contractile responsiveness of arteries to phosphodiesterase was then measured at 100 mm Hg pressure in the presence of 3 μmol/L nifedipine and correlated to the previously measured ability of the arteries to constrict in response to pressure.

In other experiments, PDC in response to a 100 mm Hg pressure step was measured, and subsequently the PKC inhibitors staurosporine (40 μmol/L), chelerythrine (12 μmol/L), and bisindolylmaleimide (5 μmol/L) or vehicle (10 μL dimethyl sulfoxide into 10.8 mL bath) were introduced. After 20 minutes, the ability of the MCAs to elicit PDC in response to the same pressure step was remeasured. Subsequently, at 100 mm Hg pressure, 3 μmol/L nifedipine was introduced into the bath (which maximally vasodilated the arteries), and the ability of the same arteries to constrict in response to 1 μmol/L phosphodiesterase in the presence of nifedipine and each of the above PKC inhibitors was measured. Using other SHRsp, we tested the ability of the MCA to constrict in response to 0.12 μmol/L vasopressin at 100 mm Hg in the presence and absence of 3 μmol/L nifedipine. All chemicals used in the study were purchased from Sigma Chemical Co with the exception of chelerythrine, which was purchased from Alexis Biochemical Corporation.

**Statistical Analysis**

Results were considered significant at \(P<0.05\). Student’s \(t\) test was used to compare independent pairs of data. In the case of multiple group comparisons, 1-way ANOVA was used to determine whether a significant between-group difference existed in a given parameter. Subsequently, Dunnett’s (Table 1, control versus test groups) or Fisher’s (Figure 2) post hoc test for multiple comparisons was used to determine which groups significantly differed from each other. Phosphodiesterase dose-response curves (Figure 3) were subjected to whole-curve analysis with the use of a general linear model of ANOVA to determine whether the amplitudes of the curves representing differing groups of animals significantly differed from each other. The curves were also assessed to determine whether a significant differential interactive effect between dose and response occurred between the groups. A significant interactive effect with dose is usually associated with situations in which curve crossover occurs or in which significant shifts in sensitivity to dose are present. In addition, a 1-way ANOVA followed by Fisher’s post hoc test was used to assess differences between groups at each applied dose. Regression analysis and the calculation of a Pearson product of correlation (\(r\) value) were used to determine whether a significant relationship existed between 2 parameters. Results in the study are expressed as mean±1 SE.

**Results**

Figure 1 shows the effects of the PKC inhibitor staurosporine on PDC in response to a 100 mm Hg pressure step in 10-week-old prestroke SHRsp. After a 6-minute equilibration period at near 0 mm Hg pressure, the application of a 100 mm Hg pressure step produced an \(\approx 36.7\%\) reduction in lumen diameter between 1 second and 4 minutes after the application of pressure. As shown in Figure 1, staurosporine (40 μmol/L) vasodilated the MCAs and inhibited PDC. We also tested the effects of PKC inhibitors chelerythrine (12 μmol/L) and bisindolylmaleimide (5 μmol/L) as well as vehicle (dimethyl sulfoxide, 10 μL into 10.8 mL bath) on...
PDC in response to a 100 mm Hg pressure step, and we tested the ability of the arteries to constrict in response to phorbol dibutyrate (1 μmol/L) at 100 mm Hg pressure (in the presence of 3 μmol/L nifedipine). These studies were performed in female prestroke SHRsp (fed a Prolab 3000 diet, PMI Feeds Inc). The results of the latter study are shown in Table 1. In the absence of PKC inhibitors, the magnitude of constriction in response to a 100 mm Hg pressure step (1st PDC) was comparable between the 3 groups. Pretreatment with chelerythrine or bisindolylmaleimide abolished or severely attenuated PDC in response to a second 100 mm Hg pressure step (2nd PDC), respectively. Both inhibitors abolished the ability of the arteries to constrict in response to phorbol dibutyrate in the presence of nifedipine.

The ability to constrict in response to a 100 mm Hg pressure step varied in relation to stroke development in SHRsp. As shown in Figure 2, MCAs from poststroke SHRsp lacked the ability to constrict in response to pressure and on average expanded their lumen diameter with the application of pressure. Prestroke SHRsp exhibited a reduced ability to constrict in response to pressure compared with MCAs from srSHR. Older (>12.5 weeks) prestroke SHRsp also exhibited smaller degrees of PDC in response to a 100 mm Hg pressure step than younger (<12.5 weeks) prestroke SHRsp.

MCAs sampled from srSHR and younger (<12.5 weeks) prestroke SHRsp constricted equally in response to phorbol dibutyrate in the presence of 3 μmol/L nifedipine (Figure 3). Constriction in response to phorbol dibutyrate was compromised in older (>12.5 weeks) prestroke SHRsp, while poststroke SHRsp were unresponsive to the phorbol ester. The inability of the MCAs to constrict in response to phorbol dibutyrate in the presence of nifedipine was consistently observed in MCAs sampled from poststroke SHRsp regardless of the severity or location of the brain hemorrhage.

Regression analysis indicated a significant direct relationship between the ability of the MCAs to constrict (percent decrease in lumen diameter) in response to a 100 mm Hg pressure step and the percent reduction in lumen diameter produced by phorbol dibutyrate. Negative sign signifies constriction; positive sign, expansion of lumen.

**Figure 1.** Effect of the PKC inhibitor staurosporine on PDC in response to a 100 mm Hg pressure step. The MCAs from 10-week-old SHRsp (n=4) were initially maintained at near 0 mm Hg pressure for 6 minutes and represurized to 100 mm Hg. The control response represents the degree of lumen constriction between 1 second and 4 minutes after the application of pressure. The arteries were then incubated with 40 nmol/L staurosporine for 20 minutes, and the ability of the arteries to elicit PDC was retested. The experiment was terminated by flushing the arteries with Ca²⁺-free Krebs’ saline containing 4 mmol/L EGTA to achieve maximal vasodilation.

**Figure 2.** PDC in MCAs sampled from srSHR and prestroke and poststroke SHRsp. The percent constriction of the lumen between 1 second and 4 minutes after the application of a 100 mm Hg pressure step (subsequent to equilibration to 0 mm Hg pressure for 6 minutes) was measured. PDC declined with age in prestroke SHRsp and was absent in poststroke SHRsp (n=4 srSHR, n=7 prestroke SHRsp <12.5 weeks, n=7 poststroke SHRsp >12.5 weeks, n=7 poststroke SHRsp).

**Table 1.** PDC of MCAs in Response to a 100 mm Hg Pressure Step Before and After Application of PKC Inhibitors or Vehicle and Subsequent Ability of Arteries to Constrict to Phorbol Dibutyrate (1 μmol/L) in the Presence of Nifedipine (3 μmol/L)

<table>
<thead>
<tr>
<th>PKC Inhibitor or Vehicle*</th>
<th>1st PDC Without Inhibitor or Vehicle*</th>
<th>2nd PDC With Inhibitor or Vehicle*</th>
<th>Construction to Phorbol Dibutyrate (3 μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + vehicle* (n=6)</td>
<td>−37.6 ± 2.2</td>
<td>−45.9 ± 4.6‡</td>
<td>−74.0 ± 2.5</td>
</tr>
<tr>
<td>Bisindolylmaleimide (5 μmol/L) (n=7)</td>
<td>−34.1 ± 3.7</td>
<td>−7.6 ± 5.5†‡</td>
<td>−1.41 ± 1.6†</td>
</tr>
<tr>
<td>Chelerythrine (12 μmol/L) (n=5)</td>
<td>−35.3 ± 5.8</td>
<td>+10.1 ± 3.8†‡</td>
<td>−1.47 ± 3.4†</td>
</tr>
</tbody>
</table>

*Dimethyl sulfoxide.
†P<0.05 vs control response.
‡P<0.05 vs 1st PDC.
Figure 3. MCA constriction in response to the PKC activator phorbol dibutyrate. Maximal vasodilation was produced in the MCAs with 3 μmol/L nifedipine. Under these conditions the lumen diameters of the arteries did not significantly differ. Cumulative dose-response curves to phorbol 12,13-dibutyrate were then produced. Stauroporine (40 mmol/L) inhibited constriction (when present) in response to phorbol dibutyrate. The experiment was terminated by maximally vasodilating the MCAs with Ca2+-free Krebs’ saline containing 4 mmol/L EGTA. Phorbol dibutyrate dose-response curve analysis is as follows: srSHR vs prestroke SHRsp >12.5 weeks, P<0.01; differential interaction with dose, P<0.001; srSHR vs poststroke SHRsp, P<0.001; differential interaction with dose, P<0.001; prestroke SHRsp <12.5 weeks vs >12.5 weeks, P<0.001; differential interaction with dose, P<0.001; prestroke SHRsp <12.5 weeks vs poststroke SHRsp, P<0.001; differential interaction with dose, P=NS. Sample sizes are the same as in Figure 2.

weeks) prestroke SHRsp (n=7), % PDC=0.292%, PDBC =0.20; r=0.936, P<0.001.

In the case of MCAs sampled from poststroke SHRsp, the inability to constrict in response to phorbol dibutyrate was not due to a general inability of the arteries to constrict. MCAs from prestroke and poststroke SHRsp were sampled and tested for their ability to constrict in response to a 100 mm Hg pressure step and to 0.12 μmol/L vasopressin in the presence and absence of nifedipine (3 μmol/L). The results of this experiment are shown in Table 2. It was observed that MCAs from poststroke SHRsp that lacked the ability to constrict in response to pressure readily constricted in response to vasopressin. We observed that pretreatment with 3 μmol/L nifedipine prevented constriction in response to vasopressin from occurring in MCAs sampled from poststroke SHRsp. MCAs from prestroke SHRsp, capable of eliciting PDC, differed from those of poststroke SHRsp in that they constricted in response to vasopressin in the presence of 3 μmol/L nifedipine. It would appear that vasopressin constriction of MCAs partly involves the opening of L-type Ca2+ channels. Once these channels are blocked, the MCAs of prestroke but not those of poststroke SHRsp are capable of using alternative mechanisms to elicit constriction in response to vasopressin.

Discussion

The key finding of the present study was the observation that the MCAs of SHRsp exhibited a compromised ability to constrict in response to elevated pressure before stroke development and lost this function after stroke developed. The inability to constrict in response to pressure was associated with a loss in the ability of the MCA to constrict in response to PKC activation by phorbol dibutyrate in the presence of nifedipine.

Studies have suggested that the activation of PKC contributes to the promotion of constriction in response to elevations in pressure. In rat posterior cerebral arteries, low doses of staurosporine and calphostin C, known inhibitors of PKC, have been shown to inhibit pressure-dependent tone. In studies involving the cremaster muscle vasculature, PKC inhibitors H-7 and staurosporine were shown to inhibit PDC without altering basal tone. Of particular interest, in this tissue the first- and second-order blood vessels of the vasculature, which passively expanded in response to pressure, exhibited PDC when the vasculature was exposed to the PKC activator indolactam (1 μmol/L) for 30 minutes. In the rabbit facial vein, stretch-activated constriction was shown to be potentiating by PKC activation with tetradecanoxyphorbol acetate at concentrations that did not enhance contraction in response to histamine or KCl. The role of PKC activation in promoting PDC is not fully understood. It is clear that the presence of an extracellular source of Ca2+ is essential in the production of constriction in response to pressure or stretch. In cerebral and renal blood vessels, Ca2+ entry into the smooth muscle likely takes place through L-type Ca2+ channels and cannot occur in the presence of Ca2+ channel antagonists. However, fura-2 studies of the cremaster muscle vasculature have indicated that the increase in smooth muscle intracellular Ca2+ in response to constriction produced by pressure (90 to 130 cm H2O) was low (~15%), less than that observed during constriction produced by agonists such as norepinephrine (10 μmol/L) and below that which alone would be required for the activation of the contractile apparatus in the absence of other facilitating mechanisms. The above observations, along with the observation that PKC inhibitors are capable of abolishing PDC or tone, have led to the hypothesis that PKC activation, along with an influx of Ca2+ into the smooth muscle, promotes constriction in response to pressure or vascular stretch. It has been suggested that both mechanisms

TABLE 2. Vasopressin Constriction of MCAs Sampled From Prestroke and Poststroke SHRsp

<table>
<thead>
<tr>
<th>Constriction in Response to</th>
<th>Poststroke SHRsp</th>
<th>Prestroke SHRsp</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mm Hg pressure step (PDC)</td>
<td>+13.2±2.4%*</td>
<td>−36.8±2.9%*</td>
</tr>
<tr>
<td>0.12 μmol/L vasopressin in presence of 3 μmol/L nifedipine at 100 mm Hg pressure</td>
<td>−1.9±2.1%</td>
<td>−21.2±5.8%*</td>
</tr>
<tr>
<td>0.12 μmol/L vasopressin in absence of nifedipine at 100 mm Hg pressure</td>
<td>−39.0±4.5%*</td>
<td>−43.6±6.9%*</td>
</tr>
</tbody>
</table>

n=MCAs from 5 SHRsp for each measurement. Negative sign signifies constriction; positive sign, expansion of lumen.

*P<0.05 vs poststroke SHRsp.
work in conjunction and that neither mechanism (Ca^{2+} influx or PKC activation) alone is capable of producing constriction in response to pressure. Consistent with this hypothesis, recent studies involving pressurized rat posterior cerebral arteries made permeable with a-toxin have indicated that contractile sensitivity to Ca^{2+} increases under conditions in which PKC is activated by indolactam.16

Other studies involving isolated arterial smooth cells have indicated that L-type Ca^{2+} channels can be opened by PKC activation through phorbol esters under constant membrane potential conditions (voltage clamp).13 This suggests the possibility that PKC activation in smooth muscle in response to elevated pressures may be directly involved in opening L-type Ca^{2+} channels. This alternative hypothesis could also explain why the individual application of PKC inhibitors or L-type Ca^{2+} antagonists inhibits pressure-dependent tone in cerebral blood vessels.5,7,8

The signal transduction pathways that lead elevations in pressure to PKC activation are not known. However, it has been demonstrated that in addition to PKC inhibitors, the inhibition of phospholipase C with U-73122 also promotes an inhibition of pressure-dependent tone in posterior cerebral arteries.17 More recently, the products of phospholipase C cleavage of phosphoinositides, inositol triphosphate, and the PKC activator diacylglycerol have been shown to increase in the smooth muscle of pressurized renal arteries.18 It is therefore possible that elevations in pressure may promote PKC activation through an increase in phospholipase C activity.

None of the experiments in the present study can definitively verify that PDC is in fact mediated by PKC activation. However, the observation that a strong quantitative relationship exists between the ability of the MCAs of SHRsp to constrict in response to pressure and to contract in response to PKC activation by phorbol dibutyrate is consistent with such a possibility. Studies have shown that a variety of phorbol esters, including phorbol dibutyrate, activate different isozymes of PKC by binding to the C1 region of the enzymes.19 This is also the site through which diacylglycerol activates PKC. If diacylglycerol stimulation of PKC is involved in promoting constriction in response to pressure, the inability of phorbol dibutyrate to promote constriction in the MCA of SHRsp may reflect the presence of a defect in the ability of the PKC system to be activated by endogenously formed diacylglycerol. Such a defect could contribute to the loss of PDC in the MCAs of SHRsp.

A unique observation made in the study was that in the presence of L-type Ca^{2+} channel blockade with nifedipine, constriction in response to vasopressin only occurred in MCA sampled from pre-stroke SHRsp capable of constricting in response to pressure and not in arteries sampled from post-stroke SHRsp that lacked this ability. Many studies, including those of cerebral arteries, have demonstrated that constriction in response to vasopressin (V_{1} vascular receptors) involves phospholipase C activation associated with an inositol triphosphate–mediated release of sarcoplasmic Ca^{2+} and diacylglycerol-mediated stimulation of PKC.20 An inability to constrict in response to vasopressin in the presence of nifedipine could reflect the presence of a defect in the above signal transduction pathways. The above finding is consistent with the possibility that a defect in the ability of diacylglycerol to activate PKC may exist in the MCA of poststroke SHRsp.

A defective PKC system is not the only abnormality observed in the MCAs of poststroke SHRsp. In preliminary reports,21 we have shown that unlike the MCAs of pre-stroke SHRsp, those of poststroke animals do not exhibit pressure-dependent smooth muscle depolarization in response to elevations in pressure but rather maintain a depolarized smooth muscle resting membrane potential that does not significantly differ between pressures of 0 and 120 mm Hg. Furthermore, in other studies (J.S. Smeda, PhD, and S. King, Msc, unpublished data, 1998), we have observed that elevations in [K^{+}], promote smooth muscle depolarization in the MCAs of poststroke SHRsp but cannot constrict the arteries. This suggests that a defect in the voltage-gated opening of the L-type Ca^{2+} channel may also exist. It has been suggested that pressure-dependent depolarization of cerebrovascular smooth muscle may contribute to the influx of Ca^{2+} through the opening of voltage-gated Ca^{2+} channels.3,22 If an influx of Ca^{2+} through L-type Ca^{2+} channels in combination with PKC activation is involved in promoting constriction to pressure, a defect in the ability of the MCA to elicit pressure-dependent depolarization and the absence of a voltage-gated influx of Ca^{2+} through L-type Ca^{2+} channels would also contribute to the loss of PDC observed in the arteries.

A compromise in the ability of the MCA to constrict in response to pressure under hypertensive conditions may have important consequences in relation to stroke development. The loss of this blood flow autoregulatory mechanism could lead to the overperfusion of the more distal segments of the middle cerebral vasculature and lead to an increase in the transmural pressures experienced by the microvasculature. Such changes could facilitate the production of cerebral hemorrhage.

Acknowledgments
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References
This article clearly demonstrates that pressure-dependent activation of rat middle cerebral arteries is dependent on functional activation of protein kinase C (PKC). The authors provide compelling data to suggest that in stroke-prone SHRsp there is inability of cerebral arteries to contract to activation of PKC by phorbol esters. Similarly, there is inability for pressure to induce activation of cerebral arterial muscle. The conclusion of the authors was that normal pressure-dependent activation of cerebral arterial muscle requires activation of PKC and that in SHRsp, pressure does not activate arterial muscle because of an inability of pressure to activate PKC. The hypothesis stated by the authors is a logical extension of the data, even though the PKC inhibitors used are not terribly specific. The fact that multiple PKC inhibitors have the same affect somewhat lessens the concern regarding inhibitor specificity.

The physiological and/or pathological significance of these findings lies in the strong suggestion that pressure-dependent autoregulation of cerebral blood flow is a PKC-dependent mechanism. The findings reported in this study are not new; however, they strongly support previous studies demonstrating a role for PKC in autoregulation of blood flow. The data suggest that there is either a genetic inability of cerebral arteries to respond to pressure or an inability of PKC to respond to a pressure stimulus. The latter is most likely, in that PKC is a common second messenger responsible for activation via a variety of neurotransmitters, paracrine and autocrine substances. A total lack of PKC activity would result in catastrophic consequences well before a stroke developed. It is, in fact, puzzling how these animals live as long as they do given the fact that they are unable to develop myogenic tone even before arterial pressure is elevated. From this perspective it is difficult to determine whether the stroke observed in these animals is due to their ability to autoregulate via a pressure dependent mechanism or rather due to loss of ability to compensate over time. Given the high levels of arterial pressure observed in stroke-prone animals, it is not surprising that stroke occurs. In fact, it is surprising that the stroke does not occur earlier, if indeed they are unable to respond actively to increases in arterial pressure. This latter point brings up the possibility that when pressure-dependent mechanisms are blunted, other compensatory mechanisms can function to maintain blood flow and protect against stroke.

Finally, with respect to activation of PKC as a function of arterial pressure, it is interesting to look at how pressure can alter the sensitivity to vasoactive agents which act via PKC. If increases in pressure elevate PKC activity, one might expect the sensitivity of agents that stimulate PKC to be increased. Indeed, it has been shown that in cerebral arteries subjected to increases in transmural pressure, the sensitivity to vasoactive agents is increased. If this logic is carried over to untreated hypertension in humans, one can speculate that there may be augmented responses to normal vasoactive stimuli which might reflect some of the pathology associated with this particular disease state.

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Reference
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