The effect of subarachnoid hemorrhage on mechanisms of vasodilation mediated by cyclic adenosine monophosphate

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Object. This study was designed to determine whether subarachnoid hemorrhage (SAH) affects the function of the K⁺ channels responsible for relaxation of canine cerebral arteries in response to adenylate cyclase activation.

Methods. The effect of K⁺ channel inhibitors on the arterial relaxation response to forskolin, a direct adenylate cyclase activator, was studied in rings of basilar arteries obtained from normal dogs and dogs in which SAH was induced (double-hemorrhage model). The levels of adenosine 3',5'-cyclic monophosphate (cAMP) were measured using the radioimmunoassay technique. In rings with the endothelium removed, relaxation induced by forskolin was not affected by SAH. The relaxation response to forskolin was reduced by charybdotoxin (10⁻⁷ mol/L), a selective Ca²⁺-activated K⁺ channel inhibitor, in normal arteries and arteries subjected to autologous blood injection. This inhibitory effect of charybdotoxin was significantly greater in arteries involved in SAH than in normal vessels. The relaxation response to forskolin was reduced by 4-aminopyridine (10⁻⁷ mol/L), a delayed rectifier K⁺ channel inhibitor, only in arteries involved in SAH. In contrast, the relaxation response to forskolin was not affected by glyburide (10⁻³ mol/L), an adenosine 5'-triphosphate–sensitive K⁺ channel inhibitor, in both normal and SAH arteries. Forskolin (3 × 10⁻⁷ mol/L) produced an approximately 10-fold increase in levels of cAMP. The basal values and increased levels of cAMP detected after stimulation with forskolin were no different in normal arteries and those exposed to SAH.

Conclusions. These results demonstrate that formation of cAMP and the relaxation response to adenylate cyclase activation are not affected by SAH. However, in diseased arteries, K⁺ channels assume a more important role in the mediation of relaxation response to forskolin, indicating that SAH may change the mechanisms responsible for vasodilation induced by cAMP.

Key Words • adenylate cyclase • cyclic adenosine monophosphate • forskolin • subarachnoid hemorrhage • cerebral vasospasm • potassium channel inhibitor • dog

Subarachnoid hemorrhage (SAH) impairs the function of cerebral arteries and frequently produces vasospasm in affected patients. Because vascular tone is regulated by the balance of vasodilator and vasoconstrictor systems, impaired arterial relaxation function can be an important factor in the pathogenesis of cerebral vasospasm. Relaxation of blood vessels is believed to be mediated by several different pathways. Activation of adenylate cyclase accompanied by subsequent accumulation of adenosine 3',5'-cyclic monophosphate (cAMP) is a potent vasodilator mechanism that may be activated by numerous endogenous stimuli, including prostacyclin, β-adrenergic agonists, calcitonin gene–related peptide (CGRP), and vasoactive intestinal polypeptide. Another major vasodilator mechanism is dependent on the enzymatic activation of guanylate cyclase and formation of guanosine 3',5'-cyclic monophosphate (cGMP). Endothelium-dependent relaxation mediated by nitric oxide (NO), which activates guanylate cyclase and thereby produces vascular relaxation, is reportedly impaired in cerebral arteries affected by SAH. However, few studies have attempted to clarify the effect of SAH on cAMP-dependent mechanisms of relaxation. Recent evidence indicates that several types of K⁺ channels, including Ca²⁺-activated K⁺ channels, adenosine 5'-triphosphate (ATP)–sensitive K⁺ channels, delayed rectifier K⁺ channels, and inward rectifier K⁺ channels, play important roles in hyperpolarization and relaxation of vascular smooth muscle. It has also been reported that cyclic nucleotides can modulate the open probability of K⁺ channels in various blood vessels, including cerebral arteries. In our previous study we demonstrated that SAH enhances the role of K⁺ channels in the cerebral arterial relaxation response to guanylate cyclase activation triggered by NO donors. However, the role of cAMP in the regulation of K⁺ channels in arteries that have developed vasospasm has not been studied. Therefore, this study was designed to determine the effect of K⁺ channel inhibitors on the relaxation response to forskolin, a direct adenylate cyclase activator, in basilar arteries (BAs).
obtained from normal dogs and those in which SAH was induced.

Materials and Methods

Experimental Model of SAH

Mongrel dogs of either sex weighing between 12 and 18 kg were used for the experiments (14 dogs with induced SAH and 17 control animals). Induction of SAH followed by cerebral vasospasm was performed as described previously. The dogs under general anesthesia induced by 15 mg/kg of thiopental sodium were intubated intravenously, the cisterna magna was aseptically punctured with a No. 22 spinal needle and 5 ml of cerebrospinal fluid was aspirated. Subsequently, 5 ml of autologous venous blood was gently injected through the spinal needle over a 2-minute period. After 15 minutes in the head-down position, the animal was allowed to recover. Two days later (Day 2), venous blood was again injected into the cisterna magna in the same manner. Seven days after the first injection (Day 7), the animals were killed to isolate their BAs. Because the identical procedures evoked reproducible vasospasm (diameter of BA on Day 7 = 57.7% of diameter before intracisternal injection of blood [six dogs]), angiography was not performed in this study. All procedures and handling of the animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Foundation.

In Vitro Studies

The brains were removed from dogs (normal animals and those exposed to SAH) that had been anesthetized with 30 mg/kg of pentobarbital administered intravenously and were placed in cold modified Krebs-Ringer bicarbonate solution (control solution) of the following millimolar composition: NaCl, 118.3; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25; Ca-ethylenediaminetetraacetic acid, 0.026; glucose, 11.1. Experiments were performed on 4-mm-long rings of BAs with the endothelium removed to eliminate the influence of guanylate cyclase activation by endothelium-derived NO. The arterial rings were placed in the control solution, and the endothelium was removed mechanically by gentle rubbing of the intimal surface with a stainless-steel wire (31-gauge diameter). Each ring was connected to an isometric force-displacement transducer and suspended in an organ chamber filled with 25 ml of control solution (37°C, pH 7.4) aerated with 94% O₂/6% CO₂. Iso metric tension was recorded continuously. The arterial rings were allowed to stabilize at a resting tension of 0.2 to 0.4 g for 1 hour. Each ring was then gradually stretched to the optimal point of its length/tension curve (approximately 3 g) as determined by the contraction induced by 10⁻⁵ mol/L of uridine 5'-triphosphate (UTP). The successful removal of the endothelium was verified by the absence of relaxation induced by 10⁻⁴ mol/L of bradykinin.

Radioimmunoassay of cAMP

A radioimmunoassay technique was used to determine the tissue content of cAMP. Arterial rings with the endothelium removed were initially incubated in the control solution, which was bubbled with 94% O₂/6% CO₂ gas mixture and maintained at 37°C. After 1 hour, the rings were incubated for an additional 30 minutes in a solution containing 10⁻² mol/L, 3-isobutyl-1-methylxanthine (IBMX) to inhibit the degradation of cyclic nucleotides by phosphodiesterases. Forskolin (3 × 10⁻⁷ mol/L) was added after 20 minutes of incubation and was in contact with the arterial rings for the remaining 10 minutes. After the incubation, the rings were immediately removed from the solution and frozen in liquid nitrogen. Cyclic AMP radioimmunoassay kits were used to make the measurements. The protein assay was conducted using an assay kit.

Pharmacological Agents

The following pharmacological agents were used: UTP, bradykinin, forskolin, diltiazem hydrochloride, charybdotoxin, glyburide, 4-aminopyridine, papaverine hydrochloride, and IBMX. The drugs were dissolved in distilled water, and volumes of less than 0.15 ml were added to the organ chambers. Stock solutions of forskolin and glyburide were prepared in dimethyl sulfoxide. Concentrations of all drugs are expressed as the final molar (moles/liter) concentration in the control solution. The arterial rings were contracted with 10⁻⁴ mol/L of UTP before the addition of vasodilating agents. Concentration–response curves were obtained in a cumulative fashion. Several rings prepared from the same artery were studied in parallel, and a concentration–response curve was established for each preparation. The relaxation response was expressed as a percentage of the maximal relaxation induced by 3 × 10⁻⁴ mol/L of papaver ine. The K⁺ channel inhibiting drugs were added 20 minutes before obtaining the concentration–response curve for each vasodilating agent. Charybdotoxin (10⁻⁷ mol/L), 4-aminopyridine (10⁻⁴ mol/L), and glyburide (10⁻³ mol/L) were used to inhibit the activation of Ca²⁺-activated, delayed rectifier, and ATP-sensitive K⁺ channels, respectively. Charybdotoxin (10⁻² mol/L) and 4-aminopyridine (10⁻³ mol/L) caused contractions of quiescent BAs; however, these contractions were not significantly different in arteries obtained from normal dogs and those from dogs exposed to SAH (Fig. 1). In addition, because UTP produced only small contractions in the rings already treated with K⁺ channel inhibitors, the absolute values of tension recorded before adding vasodilating agents did not differ significantly between control arteries and vessels treated with K⁺ channel inhibitors. In experiments with diltiazem, the median effective concentration (EC₅₀) was calculated for each arterial ring by linear interpolation between the two concentrations that evoked responses just above and below 50% of the maximum.

Statistical Analysis

The results of this study are expressed as the means ± the standard error of the mean (SEM). Statistical evaluation of the data was assessed using repeated-measures analysis of variance (ANOVA) with Bonferroni/Dunn’s post-hoc test. Statistical significance was accepted at a probability level of less than 0.05.

Sources of Supplies and Equipment

The force-displacement transducer (model F503) was acquired from Grass Instrument Co., Quincy, MA, the cAMP radioimmunoassay kit from Amersham, Arlington Heights, IL, and the DCC Protein Assay Kit from BioRad, Hercules, CA. The following pharmacological agents were purchased from Sigma Chemical Co., St. Louis, MO: UTP, bradykinin, forskolin, diltiazem hydrochloride, charybdotoxin, papaverine hydrochloride, and IBMX. Dimethyl sulfoxide, which was used as a vehicle, was also purchased from Sigma. The glyburide and the 4-aminopyridine were purchased from BIOMOL, Plymouth Meeting, PA and Research Biochemicals International, Natick, MA, respectively.

Results

Effect of SAH on Relaxation Induced by Forskolin

During contractions induced by UTP (10⁻⁵ mol/L), forskolin administration (10⁻⁵–10⁻³ mol/L) caused concentration-dependent relaxation in rings of canine BAs that lacked endothelium. The relaxation responses were almost identical in rings obtained from normal animals and dogs in which SAH was induced (Fig. 2).

Effect of K⁺ Channel Inhibitors on Relaxation Induced by Forskolin

Charybdotoxin (10⁻⁷ mol/L) reduced the relaxation response to forskolin in arterial rings obtained from both control and SAH-induced dogs (Fig. 3 left and center). However, the reduction of the relaxation response caused by charybdotoxin administration was significantly greater in SAH-involved arteries than in normal vessels (Fig. 3 right).
The administration of 4-aminopyridine (10^-3 mol/L) did not affect the relaxation response to forskolin in normal arteries (Fig. 4 left); however, it significantly reduced this response in arteries obtained from SAH-induced dogs (Fig. 4 right). Glyburide (10^-5 mol/L) did not affect the relaxation response induced by forskolin in either normal or SAH-exposed arteries (Fig. 5).

The maximal relaxation response induced by diltiazem (10^-4 mol/L) was not affected by charybdotoxin (10^-7 mol/L) or 4-aminopyridine (10^-3 mol/L) in arteries obtained from normal and SAH-induced dogs. Interestingly, these K^+ channel inhibitors augmented the relaxation response to diltiazem and decreased the EC_50 value only in SAH-exposed arteries (Table 1).

Effect of SAH on cAMP Production

In canine BA rings with the endothelium removed, forskolin administration (3 × 10^-7 mol/L) produced an approximately 10-fold increase in levels of cAMP. The basal level of cAMP and the production of this substance stimulated by forskolin were no different in normal and SAH-involved arteries (Fig. 6).

Discussion

The major finding of this study is that SAH augments the role of K^+ channels in the relaxation response to adenylate cyclase activation triggered by forskolin. A selective large-conductance Ca^{++}-activated K^+ channel inhibitor, charybdotoxin caused a significantly greater reduction of relaxation response to forskolin in arteries exposed to autologous blood injection compared with untreated vessels. This result demonstrates that the contribution of Ca^{++}-activated K^+ channels to the relaxation response following adenylate cyclase activation is enhanced in cerebral arteries exposed to SAH.

Activation of adenylate cyclase and guanylate cyclase are regarded as major mechanisms in vascular smooth-muscle relaxation. It has been reported in several studies that the basal level of cGMP and its increased production stimulated by endogenous and exogenous NO are decreased in cerebral arteries after SAH.22,32,36 In contrast, the effect of experimental SAH on the levels of cAMP has not been clearly characterized. In rabbit BAs with intact endothelium, the basal level of cAMP was increased after SAH; however, the production of cAMP in response to vasoactive intestinal polypeptide was suppressed.48 In another study performed on rabbit BAs it has been reported that basal levels of cAMP and forskolin- or CGRP-induced formation of cAMP were not affected by SAH.46 Our results in canine BAs lacking endothelium are in agreement with findings reported by Sutter, et al., indicating that in cerebral artery smooth-muscle cells, activation of adenylate cyclase and formation of cAMP are maintained after SAH. This is also consistent with the previous findings in human cerebral arteries demonstrating that prostacyclin-induced relaxation response (predominantly mediated by cAMP) are preserved during SAH.45 These findings do not necessarily have immediate clinical and therapeutic implications. Cerebral vasospasm is a complex phenomenon, and the role of cyclic nucleotides in the pathogenesis of blood-induced arterial narrowing remains to be fully characterized.

The relaxation responses induced by forskolin were almost identical in BAs obtained from normal and SAH-induced dogs. This finding is consistent with the results of cAMP measurements. However, despite the same magni-
tude of relaxation, the mechanism underlying forskolin-induced relaxation response was apparently changed by SAH. Charybdotoxin- and 4-aminopyridine–sensitive components of the relaxation response to forskolin were significantly enhanced in vasospastic arteries. Charybdotoxin and 4-aminopyridine reduced the relaxation response to forskolin but did not inhibit the response to a Ca\(^{++}\) channel antagonist, diltiazem, demonstrating selectivity of K\(^{+}\) channel inhibitors. It is interesting to note that the relaxation response to diltiazem was potentiated in SAH-affected arteries exposed to charybdotoxin and 4-aminopyridine. However, the mechanism responsible for the potentiation of the diltiazem-induced relaxation response remains to be determined.

Charybdotoxin reduced the relaxation response to forskolin even in normal BAs, indicating that Ca\(^{++}\)-activated K\(^{+}\) channels play a physiological role in the cerebral arterial relaxation response to adenylate cyclase activation. Cyclic AMP–dependent protein kinase hyperpolarizes arterial smooth-muscle cells through Ca\(^{++}\)-activated K\(^{+}\) channels, and dilation of rabbit cerebral arterioles evoked by adenylate cyclase activation is mediated by these same channels.\(^{47}\) Our results are in agreement with these studies, and our observations also indicate that Ca\(^{++}\)-activated K\(^{+}\) channels may regulate cAMP-dependent relaxation responses in large cerebral arteries. In contrast, a delayed rectifier K\(^{+}\) channel inhibitor, 4-aminopyridine, reduced the relaxation response to forskolin only in

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**Fig. 3.** Graphs showing concentration–response curves after forskolin administration in canine BAs lacking endothelium that were obtained from normal (N, left) and SAH-induced dogs (center) in the absence (control) and presence of charybdotoxin (CTX, 10\(^{-7}\) mol/L), and (right) comparison of the effect of charybdotoxin on the relaxation response to forskolin in normal and SAH-involved arteries. The relaxation response was measured during contractions induced by UTP (10\(^{-5}\) mol/L). Data are shown as the means ± the SEM and are expressed as the percentage of the maximal arterial relaxation response induced by papaverine (3 x 10\(^{-4}\) mol/L; 100% = 5.3 ± 0.3 g [seven dogs] and 5.4 ± 0.6 g [nine dogs] for arterial rings from normal dogs with and without charybdotoxin, and 4.6 ± 0.7 g [six dogs] and 4.6 ± 0.2 g [14 dogs] for arterial rings from SAH-induced dogs with and without charybdotoxin, respectively). †S.D. = significant difference between concentration–response curves (p < 0.05 by repeated-measures ANOVA).

**Fig. 4.** Graphs showing concentration–response curves after forskolin administration in canine BAs lacking endothelium obtained from normal (N, left) and SAH-induced dogs (right) in the absence (control) and presence of 4-aminopyridine (4-AP, 10\(^{-3}\) mol/L). Relaxation measures were obtained during contractions induced by UTP (10\(^{-5}\) mol/L). Data are shown as the means ± the SEM and are expressed as the percentage of the maximal arterial relaxation response induced by papaverine (3 x 10\(^{-4}\) mol/L; 100% = 5.8 ± 0.4 g [seven dogs] and 5.4 ± 0.6 g [nine dogs] for arterial rings from normal dogs with and without 4-aminopyridine, and 4.9 ± 0.4 g [six dogs] and 4.6 ± 0.2 g [14 dogs] for arterial rings from SAH-induced dogs with and without 4-aminopyridine, respectively). †S.D. = significant difference between concentration–response curves (p < 0.05 by repeated-measures ANOVA).
arteries affected by SAH, indicating that delayed rectifier K+ channels may contribute to the relaxation response induced by forskolin in SAH-involved vessels. However, because the effect of 4-aminopyridine may not be highly selective for these K+ channels, it remains to be determined whether delayed rectifier K+ channels participate in the mediation of the relaxation response to adenylate cyclase activation after SAH.

The ATP-sensitive K+ channels are thought to be important mediators of cAMP-dependent vascular relaxation. Studies in which patch-clamp techniques are used show that activation of a cAMP-dependent protein kinase increases the open probability of ATP-sensitive K+ channels in vascular smooth muscle.27,37 Furthermore, it has also been demonstrated that in rat BA vasodilation following activation of adenylate cyclase caused by forskolin was inhibited in part by glybenclamide (glyburide),24 a selective ATP-sensitive K+ channel inhibitor.2,5,33 However, in the present study, glyburide (10−5 mol/L) did not affect the relaxation response to forskolin in both normal and SAH-involved arteries. We have confirmed that 5 × 10−6 mol/L of glyburide selectively abolished the canine cerebral arterial relaxation response to cromakalim, an ATP-sensitive K+ channel opener.23 Thus, our results indicate that in canine BA, ATP-sensitive K+ channels do not play an essential role in the relaxation response to adenylate cyclase activation. Although in recent studies it has been reported that dilation of BA caused by CGRP or an ATP-sensitive K+ channel opener was enhanced in rat and

TABLE 1
Effect of CTX and 4-AP on EC50 and maximal relaxations in response to diltiazem in BAs obtained from normal or SAH-induced dogs*

<table>
<thead>
<tr>
<th>Group</th>
<th>EC50 (–log mol/L)</th>
<th>Maximal Relaxation (%)</th>
<th>No. of Dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>6.37 ± 0.07</td>
<td>92.1 ± 1.7</td>
<td>6</td>
</tr>
<tr>
<td>CTX (10−7 mol/L)</td>
<td>6.42 ± 0.08</td>
<td>93.1 ± 2.2</td>
<td>6</td>
</tr>
<tr>
<td>4-AP (10−3 mol/L)</td>
<td>6.45 ± 0.08</td>
<td>92.7 ± 2.1</td>
<td>6</td>
</tr>
<tr>
<td>SAH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>6.32 ± 0.06</td>
<td>91.3 ± 1.9</td>
<td>8</td>
</tr>
<tr>
<td>CTX (10−7 mol/L)</td>
<td>6.56 ± 0.11†</td>
<td>92.5 ± 0.6</td>
<td>5</td>
</tr>
<tr>
<td>4-AP (10−3 mol/L)</td>
<td>6.69 ± 0.08†</td>
<td>93.1 ± 0.7</td>
<td>5</td>
</tr>
</tbody>
</table>

* Relaxation measures were obtained during contractions induced by UTP (10−5 mol/L). Relaxation values are expressed as the percentage of the maximal relaxation response induced by papaverine (3 × 10−4 mol/L). Values are expressed as the means ± the SEM. Abbreviations: CTX = charybdotoxin; 4-AP = 4-aminopyridine.
† Statistically different from control SAH-induced animals (p < 0.05).

Fig. 5. Graphs showing concentration–response curves after forskolin administration in canine BAs lacking endothelium that were obtained from normal (N, left) and SAH-induced dogs (right) in the absence (control) and presence of glyburide (GL; 10−5 mol/L). Relaxation measures were obtained during contractions induced by UTP (10−5 mol/L). Data are shown as the means ± the SEM and are expressed as the percentage of the maximal arterial relaxation response induced by papaverine (3 × 10−4 mol/L; 100% = 5.6 ± 0.4 g [six dogs] and 5.4 ± 0.6 g [nine dogs] for arterial rings from normal dogs with and without glyburide, and 3.8 ± 0.3 g [six dogs] and 4.6 ± 0.2 g [14 dogs] for arterial rings from SAH-induced dogs with and without glyburide, respectively.

Fig. 6. Bar graph showing effect of forskolin (3 × 10−7 mol/L) on cAMP production in canine BAs lacking endothelium obtained from normal dogs and those in which SAH was induced. Values are expressed as the means ± the SEM (seven animals). N.S. = no significant difference.
rabbit models of SAH, the enhanced role of ATP-sensitive K* channels in the relaxation response to adenylyl cyclase activation could not be detected in canine BAs.

Our previous study has demonstrated that the role of Ca²⁺-activated and delayed rectifier K* channels in the cerebral arterial relaxation response to an NO donor (a guanylate cyclase activator) were augmented in canine BAs affected by SAH. The relaxation response caused by an NO donor, 3-morpholinosydnonimine, was reduced by charybdotoxin and 4-aminopyridine in a manner similar to that observed in the forskolin-induced relaxation response. Therefore, it is likely that SAH may modify the role of K* channels in the cerebral arterial relaxation response by affecting the common pathway after production of cyclic nucleotides. It has been demonstrated in previous studies that the exposure of cerebral arteries to hemolysate or experimental SAH causes reduction in cell membrane K* conductance, followed by depolarization of smooth muscle. Because the active state and sensitivity of arterial muscle are regulated by K* conductance, such altered muscle membrane properties may be one of the factors responsible for the increased contribution of K* channels to the cerebral arterial relaxation response. Despite the enhanced K* channel function, the relaxation responses to forskolin were not potentiated in arteries affected by SAH, indicating that SAH may also have a certain inhibitory effect on the mechanism(s) other than activation of K* channels responsible for cAMP-mediated arterial relaxation. Further analyses will be required to determine the precise mechanisms underlying the alteration of vasodilatory functions after SAH.

Conclusions

This study demonstrates that the cerebral arterial relaxation response and the formation of cAMP after adenylyl cyclase activation are not affected by SAH. However, in diseased arteries, K* channels assume a more important role in the mediation of the relaxation response to forskolin, indicating that SAH may change the mechanisms responsible for vasodilation induced by cAMP.

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