Ephedrae spamm is a major complication in patients with subarachnoid hemorrhage (SAH) and is both slow to develop and long lasting. Despite numerous studies, the mechanism of prolonged cerebral vasoconstriction is unclear. It is generally accepted that cerebral vasospasm is closely related to the release of breakdown products from the blood clot. Among these products, hemoglobin has been the focus of investigation in part because the occurrence of chronic cerebral vasoconstriction in the absence of hemoglobin is extraordinarily rare. Hemoglobin increases the tension of cerebral arteries in vitro. However, some data indicate that multiple substances contribute to vasospasm. It has been reported that the contractile response of rabbit basilar artery (BA) to purified hemoglobin was significantly weaker than that to red blood cell hemolysate. Furthermore, hemoglobin alone did not cause cerebral vasospasm in baboons.

In support of the idea that multiple spasmogens are involved in the genesis of vasospasm, we found that application of hemolysate from canine red blood cells to rat smooth-muscle cells produced a characteristic and reversible transient peak followed by sustained plateau increase in intracellular free Ca²⁺ concentration ([Ca²⁺]). This response could be reproduced by application of low-molecular-weight (LMW) fraction of hemolysate, whereas the high-molecular-weight (HMW) fraction did not increase [Ca²⁺]. The active substance in fresh hemolysate was identified as adenosine triphosphate (ATP). Our results indicated that if vasospasm is related to smooth-muscle contraction secondary to increased [Ca²⁺], then compounds in hemolysate in addition to hemoglobin may be involved.

Characteristic features of vasospasm are its delayed development 3 to 4 days after SAH and its protracted course. However, in most studies, hemolysate obtained from fresh

Effects of erythrocyte lysate of different incubation times on intracellular free calcium in rat basilar artery smooth-muscle cells

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Object. The purpose of this study was to characterize substance(s) in the erythrocytes that increase intracellular free Ca²⁺ concentration ([Ca²⁺]) in smooth-muscle cells and that therefore may be involved in the pathogenesis of vasospasm.

Methods. Because vasospasm occurs days after subarachnoid hemorrhage (SAH), the authors studied the effects of aged human erythrocyte hemolysate and its low-molecular-weight (LMW) and high-molecular-weight (HMW) fractions on [Ca²⁺], in freshly isolated rat basilar artery smooth-muscle cells. Fresh hemolysate (Day 0) produced a biphasic response consisting of a transient peak and a sustained plateau increase in [Ca²⁺], whereas hemolysate prepared from cells incubated for 3, 7, or 14 days induced only a transient response without a sustained phase. The effect of hemolysate declined with increasing incubation time. The HMW fraction and purified human oxyhemoglobin (OxyHb) did not evoke a response. The LMW fraction from Days 3, 7, or 14 produced no response at low concentrations (<10%) and a transient response at high concentrations (>20%), and the effect diminished with increasing incubation time. Unfractionated hemolysate or the LMW fraction of hemolysate incubated for 21 days produced no response. The combination of the 10% LMW fraction from Day 3 plus the 10% HMW fraction (Days 3, 7, 14, or 21) transiently increased [Ca²⁺]. However, [Ca²⁺] was not changed by the 10% LMW fraction from Day 14 plus the 10% HMW fraction from Day 3 or 14. In the presence of OxyHb, [Ca²⁺] was increased by the 10% HMW fraction on Days 3 and 7, but not by the LMW fraction from Days 14 or 21.

Conclusions. The decline over time in the effect of hemolysate on [Ca²⁺] indicates either that the time that substances are released from erythrocytes is important in the generation of vasospasm or that this experimental system as used is not representative of conditions present after SAH. The data indicate that the ability to elevate [Ca²⁺] in smooth-muscle cells with hemolysate is provided by multiple substances, including OxyHb. These substances may interact during specific times after incubation of erythrocytes in vitro.

Key Words • hemolysate • calcium • oxyhemoglobin • smooth-muscle cell • subarachnoid hemorrhage • vasospasm • rat
erythrocytes has been used to determine the immediate effects of vasospasm on cerebral vessels. We believe that it may be more relevant to study lysates from erythrocytes that have been aged so that whatever changes occur in the days before vasospasm develops may be reproduced. If the delayed onset of vasospasm is the result of the time it takes for hemolysis to occur to a substantial degree, then vasoactive substances in fresh erythrocytes may not be important in vasospasm.

The purpose of this study was to characterize substance(s) in aged erythrocytes that increase [Ca$$^{++}$$] in smooth-muscle cells and that therefore may be involved in the pathogenesis of vasospasm. Hemolysates were prepared from human red blood cells that were incubated in vitro for times spanning that of vasospasm (0, 3, 7, 14, and 21 days). We used in vitro incubation because this method has been used before and has been shown to produce vasospasm in monkeys. The hemolysate was fractionated by size to determine the molecular weight range of the spasmogen(s). The effects of the hemolysate on [Ca$$^{++}$$] in freshly isolated rat BA smooth-muscle cells were compared using Ca$$^{++}$$ imaging. The effects of purified human oxyhemoglobin (OxyHb) alone and in combination with different hemolysate fractions on [Ca$$^{++}$$] were also studied.

Materials and Methods

Preparation of Freshly Isolated Cells

All procedures involving animals were conducted according to protocols approved by the Institutional Animal Care and Use Committee. Rat BA smooth-muscle cells were isolated as previously reported. Briefly described, Sprague-Dawley rats were anesthetized with methoxyflurane and decapitated. The BAs were placed in a solution containing (in millimoles): 130 NaCl, 5 KCl, 0.8 CaCl$$\text{2}$, 1.3 MgCl$$\text{2}$, 10 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and 5 glucose at a pH of 7.4. The arteries were cleaned of connective tissue and small side branches, cut into 0.2-mm rings and incubated in a solution containing low concentrations of Ca$$^{++}$ (in millimoles: 0.2 CaCl$$\text{2}$, 130 NaCl, 5 KCl, 1.3 MgCl$$\text{2}$, 10 HEPES, 5 glucose; pH 7.4), and containing collagenase (Type II, 0.5 g/L), elastase (Type II-A, 0.5 g/L), hyaluronidase (Type IV-S, 0.5 g/L), and deoxyribonuclease (DNase I) (0.1 g/L) for 1 hour at room temperature. The rings were washed in fresh solution containing low concentrations of Ca$$^{++}$ and containing trypsin inhibitor (0.5 g/L) and DNase I (0.1 g/L), and then gently triturated. The isolated cells were plated on glass coverslips and stored at 4°C (never > 10 hours) in the previously described buffer solution containing 0.8 mM CaCl$$\text{2}$, and essentially fatty acid–free bovine serum albumin (2 g/L).

Preparation of Hemolysate and Fractions

All procedures in humans were performed according to protocols approved by the Institutional Review Board. Preparation and incubation of erythrocytes were completed in sterile conditions. Blood was drawn from healthy human donors into heparinized tubes (final concentration of heparin, 15 U/ml) and centrifuged at 100 G for 10 minutes. The supernatant, buffy coat, and uppermost red blood cells were aspirated and discarded. Red blood cells were washed twice with Ca$$^{++}$-free buffer (150 mM NaCl and 50 mM Tris, pH 7.4) and twice with a Ca$$^{++}$-free solution containing (in millimoles): 145 NaCl, 2 CaCl$$\text{2}$, 3 KCl, 1 MgCl$$\text{2}$, 10 HEPES, 10 glucose; pH 7.4. Washed erythrocytes were resuspended in 5 Vol of this Ca$$^{++}$ solution and then either frozen immediately at -80°C or incubated in the dark at 37°C for 3, 7, 14, or 21 days. At the end of the incubation period, the red blood cells were resuspended and frozen at -80°C.

For preparation of hemolysate, erythrocytes were lyzed by three cycles of freezing at -80°C followed by thawing. Cell suspensions were frozen for a minimum of 30 minutes each time and thawed rapidly by swirling the tubes in a water bath at 37°C. As soon as a suspension thawed, it was returned to the freezer. After the final freeze-thaw cycle, the suspension was centrifuged twice at 14,000 G under cold conditions to remove cell debris. The resulting hemolysate was stored at -80°C.

The LMW (< 10 kD) and HMW (> 10 kD) fractions were prepared using a centrifugal ultrafiltration device. Residual small molecules were removed from the retained HMW fraction by passage through a desalting column. The excluded HMW fractions were pooled and reconstituted to their original volumes.

The OxyHb and methemoglobin (MetHb) concentrations of hemolysate and the HMW fraction were determined spectrophotometrically by measuring absorbance at 577 and 630 nm. High-pressure liquid chromatography was used to measure concentrations of ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP) in hemolysate as described previously.

Microflowtiorimetry of [Ca$$^{++}$$].

The buffer solution for [Ca$$^{++}$$] measurement contained (in millimoles): 145 NaCl, 2 CaCl$$\text{2}$, 3 KCl, 1 MgCl$$\text{2}$, 10 HEPES, 10 glucose (pH 7.4) adjusted with NaOH. Cells were loaded with 3 μM of fura 2-acetoxymethyl ester for 30 minutes in darkness at room temperature. After loading, the cells were rinsed and the coverslip was placed in the perfusion chamber (volume approximately 600 μl). The cells were perfused for 10 minutes before measurement of [Ca$$^{++}$$].

Digital [Ca$$^{++}$$] imaging was performed using video microfluorimetry with an intensified charge-coupled device (CCD) camera connected to a computer. Fluorescence imaging was obtained with alternate excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm through the CCD camera. Typically, four to eight frames were averaged at each wavelength to produce ratio images. Data from regions of interest were displayed in real time and logged to a hard disk.

Data Analysis

The [Ca$$^{++}$$] level is expressed by the ratio value of fluorescence intensity at 340 nm divided by that at 380 nm. The values are expressed as the mean ± standard deviation (SD). Statistical differences between the control and other groups were compared using Student’s t-test, and a probability value of less than 0.05 was considered statistically significant.

Sources of Supplies and Equipment

The centrifugal ultrafiltration device (Centriprep 10) was manufactured by Amicon, Inc., Beverly, MA. The desalting column (Econo Pack 10 DG) was purchased from BioRad, Hercules, CA. The CCD camera was obtained from Hamamatsu, Bridgewater, NJ. Reagents were obtained from the following sources: Fura 2-acetoxymethyl ester, Molecular Probes, Eugene, OR; and purified, reduced human hemoglobin A$$\text{0}$ (7.3 g/dl), Hemosol, Inc. (Etobicoke, Ontario, Canada). Collagenase (Type II), elastase (Type II-A), hyaluronidase (Type IV-S), trypsin inhibitor, DNase I, bovine serum albumin, and other chemical compounds were obtained from Sigma Chemical Co., St. Louis, MO.

Results

Effects of Hemolysate on [Ca$$^{++}$$].

Hemolysate from Day 0 produced a biphasic [Ca$$^{++}$$] response consisting of a transient increase in [Ca$$^{++}$$], followed by a sustained component. The increase was concentration dependent, with a maximal response occurring at a hemolysate concentration of 10%. Compared with hemolysate from Day 0, that from Days 3, 7, or 14 produced only a transient increase in [Ca$$^{++}$$], which slowly declined to the resting level within 3 minutes without a significant sustained phase (Fig. 1). Incubated aged
erythrocyte hemolysate did not evoke a significant increase in \([\text{Ca}^{++}]\) until the concentration increased to 2%, 4%, and 10% of hemolysate from Days 3, 7, and 14, respectively. Higher concentrations produced transient responses of the same amplitude as those induced by 10% hemolysate from Day 0. Hemolysate from Day 21 produced only a minimal response even at concentrations of up to 80% (Fig. 2).

**Effects of LMW and HMW Fractions on [Ca\(^{++}\)].**

Table 1 shows that none of the HMW fractions from erythrocytes incubated for up to 21 days produced a significant increase in [Ca\(^{++}\)], at concentrations of 10% and 100% (p > 0.05 at each time). The 10% LMW fraction from Day 0 evoked a transient increase in [Ca\(^{++}\)], followed by a sustained phase. The amplitude of the maximal response was the same as that induced by 10% hemolysate from Day 0. Hemolysate from Day 21 produced only a minimal response even at concentrations of up to 80% (Fig. 2).

**Interaction of LMW and HMW Fractions**

The activity of the components inducing the increase in [Ca\(^{++}\)], was not lost during fractionation because the original response was obtained by recombining the HMW and LMW fractions from the same incubation times. This indicated that the smooth-muscle cell responses were the result of a combined effect of HMW and LMW components. To study interactions between these fractions, we used as the starting condition a 10% concentration of either the LMW or HMW fraction from Days 3, 7, 14, or 21, which by itself did not produce a response. The 5% and 10% LMW fraction from Day 3 did not produce a significant response. In the presence of the 10% HMW fraction from Day 3, however, this concentration of LMW fraction markedly increased [Ca\(^{++}\)], from 0.61 ± 0.05 and 0.64 ± 0.04 to 0.89 ± 0.08 and 0.86 ± 0.09, respectively (p < 0.05). Concentration–response curves show that in the presence of the 10% HMW fraction, the response to the 10% LMW fraction was shifted to the left and the maximal response was increased significantly (p < 0.05, Fig. 4 right). There was no significant increase in the response induced by the 10% LMW fraction when it was combined with the 10%, 20%, 40%, or 80% HMW fractions from Day 3 (p < 0.05, Table 2). At a concentration of 10%, the HMW fractions from Days 7, 14, or 21 each potentiated the response to the 10% LMW fraction from Day 3. However, compared with the response induced by using the 10% HMW fraction from Day 3, the response to its combination with the 10% HMW fraction from Day 21 was lower (p < 0.05, Table 3 and Fig. 5). If the concentration of the HMW fraction from Day 21 was increased to 20%, the amplitude of the response induced in combination with the Day 3 10% LMW fraction was 0.82 ± 0.07 (12 cells), which was the same as that induced by the combination with the 10% HMW fraction from Day 3.

**Effect of OxyHb**

Oxyhemoglobin did not produce an increase in [Ca\(^{++}\)].

**Fig. 1.** Graph showing original tracings of the change in [Ca\(^{++}\)] in rat BA smooth-muscle cells in response to addition of hemolysate from Days 0 and 3. Traces 1, 2 and 3 represent responses to the 10% hemolysate from Day 0, and 80% and 10% hemolysate from Day 3, respectively. The hemolysate from Day 0 produced a transient increase in [Ca\(^{++}\)], followed by a sustained phase that decreased rapidly to resting levels after the hemolysate was washed out (W). Hemolysate from Day 3 produced only a transient increase in [Ca\(^{++}\)] without a sustained phase. The 80% hemolysate from Day 3 induced the same amplitude of transient response as that induced by the hemolysate from Day 0.

**Fig. 2.** Chart showing concentration–response curves of the change in [Ca\(^{++}\)] in rat BA smooth-muscle cells in response to hemolysate incubated for 0, 3, 7, 14, or 21 days (20–40 cells for each point). With increasing incubation times, the curves were shifted to the right but the maximal responses remained the same for Days 0, 3, 7, and 14. The effect of hemolysate incubated for 21 days was not significant until an 80% concentration was used, and the maximal response was greatly decreased.
at concentrations of 50, 100, and 200 μM. The [Ca++] levels before and after application of 50, 100, or 200 μM of OxyHb were 0.60 ± 0.05 (35 cells), 0.60 ± 0.04 (nine cells), 0.61 ± 0.05 (17 cells), and 0.61 ± 0.045 (nine cells), respectively. If 100 μM of OxyHb was combined with the 10% LMW fraction from Days 3 or 7, a marked transient increase in [Ca++] from Day 0 to 0.59 ± 0.05 and 0.77 ± 0.05 was observed (p < 0.01, Figs. 6 and 7), respectively. The combination of OxyHb with the 10% LMW fraction from Day 14 did not increase [Ca++] (0.62 ± 0.05, p > 0.05 compared with OxyHb alone).

Concentrations of ATP and OxyHb in Hemolysates and HMW Fractions

Table 4 shows that the concentration of ATP in hemolysate declined rapidly with erythrocyte incubation time. Hemolysate from Day 0 contained a very high concentration of ATP, which had decreased to a very low level by Day 3. Figure 8 shows the concentrations of OxyHb and MetHb in the HMW fraction as a function of incubation time.

Discussion

There are four principal findings in this study. The ability of hemolysate to increase [Ca++] in rat BA smooth-muscle cells decreased with increasing time of incubation of the erythrocytes in vitro. The HMW fraction of hemolysate or pure OxyHb was devoid of [Ca++]-elevating activity, whereas the LMW fraction was active but only on Day 0. With incubation, no fraction was active alone but activity could be restored by combining the HMW and LMW fractions or OxyHb and the LMW fraction.

Vasospasm and HMW Compounds

The effects of the Day 0 hemolysate and the Day 0 LMW fraction are due to ATP. The hemolysate from Day 0 contained a very high concentration of ATP and we have previously shown that a similar hemolysate solution prepared from canine erythrocytes produced a [Ca++] response with features identical to that caused by ATP. This response could be blocked by treating the hemolysate with ATP-hydrolyzing enzymes (H Zhang, unpublished observations) and with antagonists of P2-purinoceptors, which are the receptors on which ATP acts. Furthermore, ATP evoked a biphasic increase in [Ca++] composed of a transient peak followed by a sustained phase that is similar to the hemolysate response. The disappearance of ATP from the LMW fraction at Day 3 coincides with the loss of its effect on [Ca++] . We believe this to be the most likely explanation, although further studies in which antagonists of ATP are used will be necessary to prove this. Data obtained in vivo indicate that ATP is not involved in vasospasm, and therefore enthusiasm for further studies is minimal (RL Macdonald, unpublished observations). Most studies of other small molecules have shown that they are not present in high concentrations during vasospasm and that they are thus unlikely to be important in this complication.

Vasospasm and HMW Compounds

Our data show that the HMW fraction and OxyHb did not increase [Ca++] . These results are consistent with our previous report in which an HMW fraction of fresh hemolysate had no effect on [Ca++] . They contradict other investigations of the effects of hemoglobin on isolated cells and on arteries in isometric tension. Takanashi, et al., and Vollrath and colleagues studied cultured monkey cerebral smooth-muscle cells, in which hemoglobin that had been prepared by reduction of impure ferric human hemoglobin caused an apparent increase in [Ca++] . The differences in hemoglobin solutions are unlikely to be the only reason for the lack of effect observed in the present study because hemoglobin prepared in a similar manner did not increase [Ca++] in fresh rat cerebral smooth-muscle cells (H Zhang, unpublished observations). The method for measuring [Ca++] in the earlier studies proba-
bly assessed a different type of $[Ca^{++}]_{i}$ change than the method used currently. The type of smooth-muscle cell and the preparation and culture conditions also may influence the response. Takenaka, et al.,24 reported that OxyHb that was also prepared by reduction of an impure solution increased $[Ca^{++}]_{i}$ in rat thoracic aorta smooth-muscle cells. The $[Ca^{++}]_{i}$ measurements were obtained using a system similar to our current one. It is of interest that hemoglobin has been observed to contract smooth-muscle cells in culture and to cause ultrastructural changes. In some cases, the changes took days to occur,8,22 and $[Ca^{++}]_{i}$ was not measured simultaneously in these studies. The explanation for these differences awaits elucidation in future experiments.

In isometric tension experiments, a significant contraction of cerebral arteries was produced by hemoglobin alone.5,19,25,27,31 Hemoglobin has multiple potential mechanisms by which it may contract arteries, and some of these may not be active in isolated cells. In tension preparations, the functions of vascular smooth-muscle cells are controlled by multiple factors, including transmitter release from the nerve terminals that are located in the adventitia of the arteries. Hemoglobin potentiates neurogenic constriction,12,13 induces the release of peptidergic neurotransmitters and modulators from the nerve terminals,5 inhibits sympathetic transmitter uptake by terminals,14 reduces the activity of nitric oxide,6 and interacts with serotonin receptors.20 These indirect effects of hemoglobin are unlikely to be active in isolated cells. Release of eicosanoids and endothelin can be stimulated by hemoglobin and may be more important in arterial preparations than in isolated cells.15

![Graph showing concentration-response curves of LMW fractions from Days 3, 7, 14, and 21 (15–40 cells for each point). The effect of the LMW fraction declined with increasing incubation time. Right: Graph showing concentration-response curves for the LMW fraction from Day 3 with and without the 10% HMW fraction from Day 3 (12–30 cells for each point). The 10% HMW fraction significantly potentiated the effect of the LMW fraction and shifted the curve to the left.](image)

**TABLE 2**

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>No. of Cells†</th>
<th>Resting Level</th>
<th>Response‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMW + 10% HMW</td>
<td>49</td>
<td>0.61 ± 0.05</td>
<td>0.89 ± 0.08</td>
</tr>
<tr>
<td>LMW + 20% HMW</td>
<td>30</td>
<td>0.60 ± 0.06</td>
<td>0.88 ± 0.07</td>
</tr>
<tr>
<td>LMW + 40% HMW</td>
<td>33</td>
<td>0.57 ± 0.05</td>
<td>0.89 ± 0.08</td>
</tr>
<tr>
<td>LMW + 80% HMW</td>
<td>15</td>
<td>0.58 ± 0.04</td>
<td>0.88 ± 0.05</td>
</tr>
</tbody>
</table>

* Values are expressed as ratios of fluorescence intensity (see Data Analysis).
† Number of cells from at least three different experiments.
‡ p < 0.05 compared with resting level.

**TABLE 3**

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>No. of Cells†</th>
<th>Resting Level</th>
<th>Response‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMW + 10% HMW</td>
<td>55</td>
<td>0.64 ± 0.04</td>
<td>0.86 ± 0.09</td>
</tr>
<tr>
<td>LMW + 20% HMW</td>
<td>12</td>
<td>0.62 ± 0.05</td>
<td>0.85 ± 0.07</td>
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<tr>
<td>LMW + 40% HMW</td>
<td>24</td>
<td>0.61 ± 0.04</td>
<td>0.81 ± 0.09</td>
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<tr>
<td>LMW + 80% HMW</td>
<td>18</td>
<td>0.61 ± 0.05</td>
<td>0.71 ± 0.09</td>
</tr>
<tr>
<td>LMW + 10% HMW</td>
<td>12</td>
<td>0.64 ± 0.03</td>
<td>0.72 ± 0.08</td>
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<tr>
<td>LMW + 20% HMW</td>
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<td>0.61 ± 0.07</td>
</tr>
<tr>
<td>LMW + 40% HMW</td>
<td>13</td>
<td>0.61 ± 0.07</td>
<td>0.62 ± 0.08</td>
</tr>
</tbody>
</table>

* Values are expressed as ratios of fluorescence intensity (see Data Analysis).
† Number of cells from at least three different experiments.
‡ p < 0.01 compared with resting level.
§ p < 0.05 compared with resting level.
Interactions Between Compounds

It was found that although the HMW fraction had no effect on $[\text{Ca}^{++}]_i$, activity equal to that of whole hemolysate could be restored by recombination with the LMW fraction. Hemoglobin may be the compound in the HMW fraction that contributes to this synergistic response. We suggest this because combining the LMW fraction with OxyHb, which is the most abundant compound in the HMW fraction, produced the same $[\text{Ca}^{++}]_i$ response. Like the HMW fraction, OxyHb produced no response when added by itself to the smooth-muscle cells. Furthermore, the ability of the HMW fraction to increase $[\text{Ca}^{++}]_i$ when combined with the LMW fraction decreased with increasing incubation time, which correlates with the decline in OxyHb content of the HMW fraction (Fig. 8). Whether other substances contribute cannot be ascertained at present. The interaction of OxyHb and LMW to produce a greater response than that seen with either compound alone has been observed before in cerebral arteries during isometric tension recording. The active substance in the incubated LMW fraction has not been identified.

One can speculate on the mechanism by which HMW and LMW compounds interact to cause the $[\text{Ca}^{++}]_i$ response. It is unlikely that there is a reaction between the two components that produces a stable third substance that causes the increase in $[\text{Ca}^{++}]_i$. If this was the case, the third substance would have already formed during the incubation, and separation into HMW and LMW fractions would simply partition the active substance into one fraction. It is possible that a third substance with a short half-life is formed. Alternatively, two or more substances may be required to activate the same or separate pathways simultaneously and both may be needed to increase $[\text{Ca}^{++}]_i$.

Transient and Plateau Phases of the $[\text{Ca}^{++}]_i$ Response

We previously demonstrated with fresh canine hemolysate from different incubation times.

<table>
<thead>
<tr>
<th>Time</th>
<th>ATP (µM)</th>
<th>ADP (µM)</th>
<th>AMP (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>170</td>
<td>62</td>
<td>17</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.2</td>
<td>0.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

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Relation to Vasospasm

The hemolysate had less effect on [Ca++]i, as incubation time lengthened, which does not correlate with the time course of vasospasm in vivo. One explanation is that vasospasm begins with hemolysis and release of the erythrocyte contents. The erythrocytes contain vasoactive substances or can incite reactions to produce such substances. Vasospasm is limited by the decay of active components with time. It should be noted that the biochemical reactions that occur with the breakdown of the erythrocytes and blood clot in vitro may be different from those that occur in the subarachnoid space after SAH. The reactions may be the same but they may occur at a different rate in vitro compared with in vivo.

The relevance to vasospasm of acute changes in smooth-muscle cell [Ca++]i, could be questioned because vasospasm occurs more than 3 days after SAH. However, in studies in which the subarachnoid clot is removed either surgically or by intracisternal fibrinolysis after the hemorrhage, it has been suggested that significant vasospasm does not occur unless the clot is present for 3 or more days.59 Thus, the processes leading to vasospasm may occur over relatively short times.

Finally, the use of [Ca++]i changes in smooth muscle to study vasospasm is based on several assumptions, including one that smooth-muscle contraction is the cause of vasospasm and that this contraction is mediated by changes in [Ca++]i. The results reported herein support these assumptions but cannot be used to exclude the possibility that vasospasm occurs by other mechanisms or that contraction develops independent of changes in [Ca++]i. These issues have been reviewed elsewhere.89

Finally, changes in [Ca++]i may correlate with signaling events leading to changes in the smooth-muscle cells that make them more responsive to other spasmogens.

Acknowledgments

We thank Hemosol, Inc. for supplying the purified human hemoglobin.

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