Altered Membrane Properties of Cerebral Vascular Smooth Muscle Following Subarachnoid Hemorrhage: An Electrophysiological Study

I. Changes in Resting Membrane Potential (\(E_m\)) and Effect on the Electrogenic Pump Potential Contribution to \(E_m\)

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**SUMMARY** Subarachnoid hemorrhage was produced experimentally in cats by intracisternal injection of non-heparinized autologous arterial blood obtained by cardiac puncture under ketamine and xylazine anesthesia. Cats were sacrificed at varying time intervals between 30 min and 7 days post ictus. Measurements of resting membrane potential were recorded from smooth muscle cells of the basilar artery. These measurements were obtained by impalement from the adventitial surface of isolated but otherwise intact segments of the artery using glass microelectrodes with tip sizes less than 0.1 \(\mu m\). The resting membrane potential recorded in vitro from animals previously subjected to subarachnoid hemorrhage in vivo was consistently and significantly depolarized when compared to normal controls. This depolarization was present as early as 30 min post ictus. Addition of the cardiac glycoside, ouabain, in a concentration of \(10^{-8}\)M depolarized cells from both control and experimental animals. There is a significant electrogenic pump potential recorded in vitro from animals previously subjected to subarachnoid hemorrhage in vivo was not, therefore, due to impairment of the electrogenic pump. The significance and implications of these findings are discussed.

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**Received July 23, 1984; revision #1 accepted March 14, 1985.**

IT HAS BECOME INCREASINGLY EVIDENT that the pathophysiology of cerebral vasospasm following subarachnoid hemorrhage (SAH) is complex and multifactorial. Numerous investigations have been undertaken to define the role of blood and/or blood products in the pathogenesis of vascular spasm, decreased cerebral perfusion and ischemia, all of which may occur clinically following SAH. Release of serotonin from platelets, prostaglandins (specifically the labile metabolite thromboxane \(A_2\)), catecholamines, thrombin, histamine, both hemoglobin and oxyhemoglobin, \(K^+\), an unidentified polypeptide and an hypothalamic extract are amongst many sub-
stances which have been putatively incriminated. The literature has been reviewed recently by White. The difficulties involved in attempting to identify a specific etiological agent in the pathogenesis of cerebral vasospasm are understandable when one considers that the literature is replete with a host of substances known to influence vascular smooth muscle (VSM) tone (see review by Bolton).

Numerous in vitro studies of the pharmacological responses of cerebral arteries from both a wide variety of animal species and human post mortem material have been undertaken. Measurements of vessel reactivity in response to various exogenously applied agents have involved either mechanical methods of measuring tension development using force transducers or optical methods of measuring changes in vessel diameter. Few electrophysiological studies have been performed on VSM following experimental SAH. VSM, in fact, presents considerable difficulties in this regard. The cells are amongst the smallest of mammalian cells (2–5 μm maximum diameter) and are embedded in a dense matrix of collagen, elastin and other tough connective tissue. They are, therefore, difficult to impale. Linear cable theory as applied to the isolated squid axon by Hodgkin and Huxley is not readily applicable to VSM which is anatomically and functionally a syncytium. The effect of SAH on the extracellular microenvironment has been investigated using ion-specific K⁺ and Ca²⁺ microelectrodes. A profound cellular depolarization was observed immediately after SAH.

The present study was undertaken to determine for the first time the effect of experimental SAH on the electrical properties of VSM cells using intracellular microelectrodes. This electrophysiological study was designed to elucidate possible mechanisms of action rather than attempting to identify any causative agents. Since the final common pathway for VSM reactivity must reside at the membrane level, biophysical events such as altered ionic conductances may be important in the production of cerebral vasospasm. Since VSM has a significant electrogenic pump potential contribution to the resting membrane potential (Eₚm), studies were also undertaken to determine the effect of the cardiac glycoside, ouabain, a potent blocker of the enzyme Na⁺, K⁺-ATPase responsible for maintaining the cationic electrochemical gradients.

**Methods**

Adult mongrel cats of either sex, weighing 2 to 5 kg were anesthetized with a combination of intramuscular ketamine 30 mg/kg and xylazine 20 mg/kg body weight. SAH was produced by slow injection of fresh autologous non-heparinized arterial blood into the cisterna magna. Cisternal puncture was performed via the posterior atlanto-occipital route using a 26 gauge, 38 mm lumbar puncture needle. 1–2 ml of cerebrospinal fluid (CSF) was slowly withdrawn and discarded. Arterial blood was obtained by direct cardiac puncture using a 21 gauge hypodermic needle. This procedure is tolerated well in the cat. Arterial blood 0.5 ml/kg body weight was then manually injected at a rate not exceeding 1 ml/min. The animals were then tilted head down for 30 min to facilitate settling of the blood around the basilar artery by gravitation. A total of 23 cats underwent production of experimental SAH (fig. 1). These animals were sacrificed at varying time intervals between 30 min and 7 days post ictus. A total of 8 cats underwent cisternal puncture with withdrawal of 1–2 ml CSF which was then reinjected. These animals served as sham controls.

All animals were killed by aortic transection. The brains were rapidly removed and the basilar arteries immediately dissected out under magnification and placed in an ice-cold physiological salt solution (PSS) of the following composition (millimolar concentrations): Na⁺/141; K⁺, 4.7; Ca²⁺, 1.6; Cl⁻, 124; H₂PO₄⁻, 1.7; HCO₃⁻, 25; and glucose, 11. Part of the preparation was used immediately and the remainder, after storage in PSS at 4°C, within 24 hr. Isolated segments of basilar artery (approximately 1.5 cm in length) distal to the origin of the anterior inferior cerebellar arteries were used. They were pinned under magnification onto a layer of agar coating the floor of a specially designed chamber using stainless steel pins. Great care was taken to ensure that the vessel segment was not subjected to any degree of longitudinal stress.

The chamber was continuously suffused with PSS containing 10 mM HEPES buffer. The solution was aerated with 95% O₂/5% CO₂ giving a pH of 7.4 and PCO₂ of 40 mm Hg. The solution was maintained at a mean temperature of 37°C; range, ± 0.5°C. A gravity feed non-recirculating system was employed since...
pump driven systems were insufficiently vibration free for the purposes of sustaining microelectrode impalements, and were also an unwelcome additional source of AC line frequency noise. The fluid level of the solution in the chamber was kept to no more than 2 mm above the vessel in order to reduce the input capacitance of the microelectrode.

All vessel segments were allowed to equilibrate for 90 min before microelectrode impalements were begun. In several experiments \( E_m \) was recorded both before and after the application of \( 10^{-5} \text{M} \) ouabain to the bathing solution. These measurements were made within 15 min so that depolarization due to ion shifts (i.e. run down of the ionic gradients) did not occur.

For measurements of \( E_m \) glass microelectrodes were used according to methods previously described. They were pulled from borosilicate single glass capillaries with an inner filament and an overall diameter of 1.2 \( \pm \) 0.1 mm. They were back filled with 3MKCl (pH 2.0), had tip resistances of 50 to 80 Mohms and tip potentials of less than 7 mV. They were mounted on a Zeiss sliding micromanipulator and interfaced to the electronics using silver/silver chloride half-cells. The ground electrode was a silver wire electrolytically chlorided to minimize polarization, i.e. capacitative coupling at the metal/electrolyte junction. A reference electrode was placed close to the vessel and membrane potentials were measured differentially. All impalements were made from the adventitial surface of isolated but otherwise intact arterial segments. The criteria for a successful impalement included no change in electrode resistance or tip potential before and after each impalement, a sharp drop in voltage upon entry of the microelectrode into the cell and a sharp return to zero upon exit, and an input resistance \( (R_i) \) of less than 60 Mohms.

Pickup and shunt capacitance were minimized by mounting the unity-gain impedance reduction stage in the preamplifier probe as close as possible to the recording electrode. The recording amplifier (high impedance electrometer) possessed capacitance neutralization, single electrode resistance measurement circuits and an internal bridge circuit. The bridge circuit was balanced prior to each microelectrode penetration. \( R_i \) was determined by applying rectangular hyperpolarizing and depolarizing current pulses \( (I_e) \) of various intensities through the microelectrode during impalement and recording the associated voltage changes \( (\Delta V) \). The current pulses were of 10 msec duration and values of \( \Delta V \) were taken when voltage displacement reached a steady state. The data from each penetration were plotted and the slope of the steady state voltage versus current \( (\Delta V/I_e) \) curve through the origin (i.e. infinitesimally small \( \Delta V \) and zero applied current) was taken as the value of \( R_i \).

Experiments were conducted on a vibration free table of grounding and the use of a Faraday cage. The data and noise interference (line AC, radio frequency and electromagnetic) kept below 500 \( \mu \text{V} \) with a minimum of grounding and the use of a Faraday cage. The data was recorded using an oscilloscope, FM tape recorder and a high frequency chart recorder. Statistical analysis was performed using a DEC PDP11 microcomputer.

The viability of the vessel segments used was assessed following each experiment by recording tension development in response to serotonin. The lumen of each vessel segment was threaded with two 32 \( \mu \text{m} \) tungsten wires. These wires were then stretched over stainless steel rings so that the vessel was mounted between the wires. One ring was secured and the other connected to a very sensitive load cell. This system can resolve as little as 0.1 mg developed force and was designed with the help of Dr. W. Halpern. A resting tension of 300 mg was applied to the vessel segment which was then allowed to equilibrate for 90 min. Contractions were recorded isometrically. Dose-response curves were obtained by a step-wise increase in concentration of serotonin (creatine sulphate complex) from \( 3 \times 10^{-9} \) to \( 10^{-5} \text{M} \). Each concentration was washed out before the next higher concentration was added. The peak tension development after each dose was considered the response in the construction of the dose-response curves. Serotonin was made up in PSS with the addition of ascorbic acid (176 mg L\(^{-1}\)) to prevent oxidation. Serotonin induced a dose-dependent contractile response of the basilar artery in normal cats. The \( ED_{50} \) was 1.2 \( \times 10^{-8} \text{M} \). Interestingly a marked increase in sensitivity to serotonin was observed following SAH.

**Results**

**Effect of SAH on \( E_m \)**

Figure 2 shows the \( E_m \) in mV (mean \( \pm \) SD) recorded in the control and experimental groups. The \( E_m \) recorded in the experimental group of animals \((n = 8)\) was \(-62.5 \pm 3.2 \text{ mV}\). A total of 60 impalements were achieved in this group. This result is comparable to the figures of \(-62 \pm 0.7 \text{ mV}\) and \(-64 \pm 1.6 \text{ mV}\) (both mean \( \pm \) SE) recorded from feline basilar arteries in earlier studies. The \( E_m \) recorded in the experimental group of animals \((n = 23)\) was \(-47.4 \pm 4.3 \text{ mV}\). A total of 349 impalements were achieved in this group. Figure 3 shows the actual distribution of values of \( E_m \) for both control and experimental groups of animals. All the VSM cells of the basilar arteries from those animals in the experimental group (i.e. following experimental SAH in vivo) were, in vitro, consistently and significantly depolarized \((p < 0.001)\). The mean depolarization recorded was 15.1 mV. This degree of depolarization was observed in all the animals in the experimental group irrespective of the time of sacrifice following SAH. Table 1 shows the \( E_m \) recorded in relation to the duration of SAH. There was no statistical difference in the degree of depolarization recorded with respect to time. These results show that VSM cells are significantly depolarized within 30 min of experimental SAH and that such depolarization persists for at least 7 days.

**Effect of Ouabain on \( E_m \) in Both Normal and Experimental Animals**

Figure 4 shows the \( E_m \) in mV (mean \( \pm \) SD) recorded in the control group of animals following the applica-
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Figure 2. Histogram comparing values of resting membrane potential (E_m) in vascular smooth muscle cells from cat basilar arteries in normal animals and in those subjected to experimental subarachnoid hemorrhage. The numbers within each column represent the number of impaled cells (numerator) and the number of different preparations (denominator). The vertical bars represent the SD for the number of cells impaled.

The mean depolarization recorded was 12.8 mV and represents the electrogenic pump potential contribution to E_m. The degree of depolarization recorded following addition of ouabain to the bathing solution is of a similar order to that seen following experimental SAH. This might imply that depolarization of VSM cells following SAH occurs as a result of inhibition of the electrogenic pump mechanism. In order to test this hypothesis a group of experimental animals (n = 6) with a mean E_m of -49.5 ± 3.2 mV (57 impalements) following SAH were similarly treated with ouabain. The E_m recorded following the application of 10^{-5}M ouabain was -39.3 ± 2.1 mV (32 impalements) (fig. 5). The mean depolarization of 10.2 mV represents the electrogenic pump potential contribution to E_m. The difference between the control and experimental groups following addition of ouabain, viz: 12.8 and 10.2 mV respectively, is not statistically significant. Clearly SAH has minimal, if any, effect upon the electrogenic pump potential contribution to the E_m of cerebral VSM cells.

Discussion

There can exist a very tight coupling between electrical and mechanical events in VSM. In rat caudal artery over 80% of total tension development is produced over only a 14 mV change, and a 4 mV depolarization can result in up to 30% of the total developed tension.32 A high degree of electromechanical coupling is related, in part, to the low slope of the curve relating

Table 1. Resting Membrane Potential (E_m) of Vascular Smooth Muscle Cells from Cat Basilar Arteries in Relation to Duration of Experimental Subarachnoid Hemorrhage

<table>
<thead>
<tr>
<th>Duration of SAH</th>
<th>No. of animals</th>
<th>No. of impalements</th>
<th>E_m ± SD (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>1</td>
<td>28</td>
<td>-50.0 ± 2.8</td>
</tr>
<tr>
<td>1 hr</td>
<td>1</td>
<td>33</td>
<td>-47.6 ± 3.2</td>
</tr>
<tr>
<td>2 hr</td>
<td>1</td>
<td>21</td>
<td>-48.2 ± 4.6</td>
</tr>
<tr>
<td>4 hr</td>
<td>1</td>
<td>16</td>
<td>-46.4 ± 3.1</td>
</tr>
<tr>
<td>12 hr</td>
<td>1</td>
<td>6</td>
<td>-42.3 ± 1.9</td>
</tr>
<tr>
<td>18 hr</td>
<td>1</td>
<td>25</td>
<td>-46.8 ± 4.4</td>
</tr>
<tr>
<td>21 hr</td>
<td>1</td>
<td>47</td>
<td>-42.8 ± 3.4</td>
</tr>
<tr>
<td>1 day</td>
<td>3</td>
<td>34</td>
<td>-47.7 ± 3.6</td>
</tr>
<tr>
<td>2 days</td>
<td>3</td>
<td>38</td>
<td>-47.9 ± 3.5</td>
</tr>
<tr>
<td>3 days</td>
<td>2</td>
<td>26</td>
<td>-51.0 ± 3.7</td>
</tr>
<tr>
<td>4 days</td>
<td>2</td>
<td>23</td>
<td>-50.7 ± 4.4</td>
</tr>
<tr>
<td>5 days</td>
<td>2</td>
<td>15</td>
<td>-48.3 ± 3.1</td>
</tr>
<tr>
<td>6 days</td>
<td>2</td>
<td>21</td>
<td>-46.4 ± 3.9</td>
</tr>
<tr>
<td>7 days</td>
<td>2</td>
<td>16</td>
<td>-47.3 ± 4.7</td>
</tr>
</tbody>
</table>
The low slope of the curve relating $E_m$ changes to $[K^+]_i$, may indicate that the development of tension in VSM is closely related to changes in $E_m$, in that even small changes in membrane voltage are capable of inducing relatively large changes in tension. A very close relationship has been demonstrated between $E_m$ and tension development in $K^+$-depolarized canine carotid arteries, with a depolarization of 6 mV causing a significant increase in tension. There is a strong correlation between $E_m$ and tension in $K^+$-depolarized rabbit pulmonary artery. Similarly, when the VSM membrane is depolarized by the application of outward current pulses, the developed tension is very closely related to changes in $E_m$, with only a 4 mV change in $E_m$ resulting in significant tension development.

Thus, the level of $E_m$ in VSM is an important modulator of tension development.

The relationship between $E_m$ and contraction medi-
ated by certain neurotransmitters is not clearly established. In many of the isolated arterial preparations studied, contraction in response to low doses (<10^{-5}M) of norepinephrine is not preceded by membrane depolarization.\textsuperscript{34, 37, 40-42} Such contraction, not associated with depolarization of VSM, is commonly referred to as pharmacomechanical coupling.\textsuperscript{43, 44} However, norepinephrine at higher doses causes the membrane to depolarize as tension increases.\textsuperscript{40-42, 45} There is clear evidence for electromechanical coupling in response to neurotransmitters in cerebral arteries. In rabbit basilar artery there is a correlation coefficient between $\Delta E_m$ and $\Delta t$ension of 0.95 in response to dopamine.\textsuperscript{46} Similar evidence for electromechanical coupling in response to serotonin and morphine has been found in cat basilar artery.\textsuperscript{31, 47} This finding with regard to serotonin is of importance in that this amine has long been incriminated in the pathophysiology of cerebral vasospasm following SAH. Increased sensitivity to serotonin has been described following SAH.\textsuperscript{48}

The final common pathway for the control of vascular reactivity and ultimately, regulation of cerebral blood flow, resides at the level of the VSM cell membrane. The processes controlling activation of the VSM cell are regulated or influenced by biophysical events occurring at the level of the plasma membrane. For example, if ionic channels are blocked or inactivated then many vasoactive agents are unable to exert their agonistic or antagonistic effects. The precise degree of membrane control involved in activation of the VSM cell as determined by corresponding changes in $E_m$ and mechanical events remains somewhat controversial largely due to the technical difficulties involved in measuring electrical and mechanical events simultaneously. However, three facts demonstrate that a high degree of activation exists at the level of the plasma membrane. First, depolarization of the VSM cell increases the influx of extracellular Ca$^{2+}$ with resultant tension development.\textsuperscript{26, 49, 50} Second, agonist stimulation of VSM either causes a change in $E_m$ or increases ionic conductances through voltage sensitive channels.\textsuperscript{29, 37, 51} Third, if the level of $E_m$ prior to agonist stimulation is changed in VSM relative to its resting value, the sensitivity to that agonist is markedly affected.\textsuperscript{50, 52, 53}

The results of this study show that VSM is significantly depolarized following experimental SAH. This depolarization was observed as early as 30 min post ictus and was evident throughout the time course of the study (i.e. up to 7 days). It would appear that $E_m$ is reset very early following SAH and since $E_m$ is clearly important in regulating the contractile state this would profoundly affect subsequent responses of VSM to a wide variety of stimuli.

Ever since Brawley's work on experimental SAH in dogs,\textsuperscript{44} cerebral vasospasm has been generally considered to be a biphasic phenomenon. The present electrophysiological study provides evidence that VSM cell depolarization is the first and almost immediate pathophysiological consequence of SAH. This raises the question of a second insult occurring subsequently leading to clinically observed cerebral vasospasm. The present model of experimental SAH (i.e. intracisternal injection of autologous arterial blood) has been used by many workers.\textsuperscript{55-58} Zervas and coworkers\textsuperscript{59} have developed a two stage model in dogs by repeating the intracisternal injection of blood at 48 hrs. The second injection led to irreversible vasospasm. Such irreversibility in experimental models is associated with light and electron microscopic changes in VSM cells leading ultimately to myonecrosis.\textsuperscript{56-57} It is tempting to speculate whether the second insult in the clinical situation represents a further bleed (as in the two stage experimental model) or occurs as a result of some time dependent breakdown product of blood present from the initial bleed. It should be emphasised that the current model is one of SAH and not one of proven cerebral vasospasm. The presence or absence of narrowing of the basilar artery was not determined angiographically. Nonetheless, the changes observed in the electrical properties of the VSM cell membrane following SAH are significant and presumably germane to the subsequent development of irreversible cerebral vasospasm. Since current therapy is uniformly unrewarding in the context of clinically established vasospasm it may well be productive therapeutically to explore the initial and presumably reversible pathophysiological consequences of SAH. Further evidence from $E_m$ versus log $[K^+]$, curves, voltage versus current curves and the development of spontaneous action potentials (Waters, unpublished data) suggests that a decreased potassium conductance ($g_k$) occurs. Absolute confirmation of such an altered ionic conductance occurring as the first pathophysiological consequence of SAH will depend upon analysis of single ion channels in isolated VSM cells.

There is a significant electrogenic pump potential contribution to the $E_m$ of VSM cells. The Na – K pump is responsible for maintaining the cation electrochemical gradients. The diffusion potentials for K$^+$ ($E_k$) and Na$^+$ ($E_{na}$) are about $-94$ mV and $+60$ mV respectively (although some estimates of $E_{na}$ are as high as $+130$ mV). Since in VSM the electrochemical potentials of K$^+$ and Na$^+$ are different from the measured resting potential, these ions must be actively transported across the cell membrane. Such an ion pump maintaining these ion gradients can be either neutral (i.e. it functions without net transfer of electrical charges across the cell membrane) or it is electrogenic when charge transfer occurs. In the latter case the pump generates a current which creates an electrogenic potential proportional to the membrane resistance. The enzyme responsible for the pump is Na$^+$, K$^+$-ATPase (ATP phosphohydrolase, E.C.3.6.1.3.).

Inhibition of Na$^+$, K$^+$-ATPase in VSM results in depolarization of the membrane.\textsuperscript{50, 56} Methods commonly used for inhibiting Na$^+$, K$^+$-ATPase include cardiac glycosides, reduction in $[K^+]$, and lowering temperature. The electrogenic contribution to $E_m$ ranges from 8–20 mV depending upon the vessel studied.\textsuperscript{56} In most vertebrate striated muscle cells the electrogenic pump potential contribution to $E_m$ is small and
of the order of 2-8 mV. Raising [K+] in a vessel has been in low K+ solutions results in a significant hyperpolarization of the membrane.65, 66 With maximal stimulation of the electrogenic pump, the membrane hyperpolarizes up to 27 mV and is accompanied by a sizable relaxation.66 The reason low [K+] stimulation is so effective probably relates to the fact that when the membrane is depolarized by inhibition of Na+, K+-ATPase in low K+ solutions, there is an increase in intracellular Na+. The increase in intracellular Na+ also acts to stimulate the Na+, K+-ATPase by saturating intracellular binding sites. This K+ induced hyperpolarization is closely linked to relaxation in arteries.65-67 and has proved to be a useful tool in the study of disease states which may involve abnormalities in arterial muscle, such as hypertension.65, 68

In the present study the electrogenic pump potential contribution to E<sub>mem</sub> was abolished by the use of the cardiac glycoside, ouabain, in a concentration of 10<sup>-7</sup>M. The results clearly indicate that the electrogenic pump mechanism is not significantly affected following SAH. Since the pump is capable of stimulation hereby pushing E<sub>mem</sub> nearer E<sub>K</sub> (effectively hyperkalemia) relaxation has been induced.65-67

The results clearly indicate that the electrogenic pump is a useful tool in the study of disease states which may involve abnormalities in arterial muscle, such as hypertension.65, 68

Acknowledgments

This work has been carried out as part of the requirement for the degree of M. Chir. of the University of Cambridge.

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Altered membrane properties of cerebral vascular smooth muscle following subarachnoid hemorrhage: an electrophysiological study. I. Changes in resting membrane potential (Em) and effect on the electrogenic pump potential contribution to Em.
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Stroke. 1985;16:990-997
doi: 10.1161/01.STR.16.6.990

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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