Cholinergic Control of Blood Flow in the Cerebral Cortex of the Rat

BY OSCAR U. SCREMIN, M.D., ALFREDO A. ROVERE, M.D., AUGUSTO C. RAYNALD, M.D., AND ADOLFO GIARDINI, M.D.

Abstract: Cholinergic Control of Blood Flow in the Cerebral Cortex of the Rat

Local blood flow was measured in the somatosensory cortex of urethanized rats by means of the hydrogen clearance method. The variations of cortical blood flow in the course of the anesthesia and the effects of the topical application of atropine, eserine and cholinomimetic drugs were studied. During urethan anesthesia, the electrical activity of the cerebral cortex fluctuated between a synchronized and a desynchronized state. During desynchronization, local cortical blood flow increased significantly. This increase in blood flow could be prevented by topical application of atropine and exaggerated by topical eserine. Topical application of arecoline, carbaminoylcholine, pilocarpine or acetylcholine with eserine significantly increased cortical blood flow. It is concluded that the increase in cortical blood flow that accompanies cortical desynchronization in the urethanized rat is mediated, at least in part, by a neurogenic mechanism that involves a cholinergic step at the cortical level.

Introduction

Cortical blood flow increases following electrocortical desynchronization.\(^1\)\(^-\)\(^4\) This effect has been frequently ascribed to the local vasodilating action of CO\(_2\) as metabolism actually increases during cortical desynchronization,\(^4\)\(^,\)\(^5\) and inhaled CO\(_2\) induces a significant vasodilation at the cerebral territory.\(^6\)\(^-\)\(^9\) In addition, the stimulation of peripheral autonomic innervation exerts minor effects over cerebral blood flow.\(^10\)\(^,\)\(^11\) However, recent reports indicate that stimulation of discrete sites within the brain stem can induce cerebral vasodilation.\(^12\)\(^,\)\(^13\) On the other hand, several reports point to the idea that the cerebrovascular action of inhaled CO\(_2\) might be indirect, as it can be blocked by transection\(^14\) or localized lesions\(^15\) of the brain stem. These facts suggest the existence of central neurogenic mechanisms in the control of cerebral blood flow, but little is known about the transmitters possibly involved. The effect of autonomic drugs on cerebral blood flow usually has been assessed after systemic administration and the results are difficult to interpret due to general hemodynamic actions and the existence of a blood-brain barrier for many of them.\(^16\) On the other hand, detection of blood flow at the carotid arteries, jugular veins, or cranial sinuses gives an average value of a heterogeneously perfused organ, masking possible regional differences. In an attempt to overcome these limitations, we set up a preparation in which blood flow was measured locally in anesthetized rats, by means of the hydrogen clearance method, at the primary somatosensory area of the cortex. Small amounts of drugs were topically applied to the area where the measurements were taken from, in order to assess their effect on blood flow. The same electrode that detected hydrogen current was used to derive the electrocortico-gram and the somatosensory response to shocks at the contralateral forepaw in order to correlate local blood flow with electrical activity.

Methods

A total of 165 inbred albino rats 200 to 300 gm body weight were used.

Stages of Urethan Anesthesia

Urethan was administered by the intraperitoneal route at doses of 1.20 to 1.80 gm/kg. Animals were tracheostomized and fixed to a nose clamp.
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Temperature was permanently monitored and maintained at 35° C by means of a heating lamp. The EEG was recorded bipolarly with stainless steel epidural screws inserted over nonexposed frontal and parietal areas. Time constant of the recording system was set at 0.1 second. Electrical activity in the dorsal hippocampus was recorded with a stainless steel needle electrode insulated except at the tip and inserted by means of "La precision cinematographique" stereotaxic instrument at coordinates A (frontal plane) 3.430 mm; L (lateral plane) 2 mm; and 2.6 mm below cortical surface, according to the König and Klippel atlas for the rat brain.17

Electrical activity of neck muscles was recorded with nichrome wires insulated except for 0.5 mm adjacent to the tip and inserted into the muscles. Respiratory frequency was recorded by means of the variation in resistance of a thermistor placed in the tracheal airway. Recording of blood pressure from internal carotid artery was performed with a Sanborn transducer Model 267-B connected to the polygraph. Arterial PCO₂ was obtained from pH measurements before and after equilibration at 38° C with a known P₁V₂ by the interpolation method.18 Measurements of blood pH were performed with a Metrohm micro blood pH electrode and a Beckman pH Research meter.

The somatosensory response to shocks at the right forepaw was recorded. To accomplish this, two stainless steel stimulating electrodes were placed at the right forepaw and square wave pulses 4 to 6 volts amplitude, 0.7 ms duration, and 0.5 cps frequency from a Grass S4 stimulator and isolation unit were delivered through them. The left cerebral cortex was then exposed and explored with a platinum surface electrode to localize the somatosensory response by use of the criterion of minimum latency. A second platinum electrode, insulated except at the tip, was inserted at the same place at a depth of 600 μm. In some experiments, unitary activity was recorded by means of extracellular glass microelectrodes, about 2 megohms impedance. The signals were amplified by a Grass P6 DC Preamplifier and displayed on a Tektronix 502 C.R.O.

STANDARDIZATION OF LOCAL BLOOD FLOW MEASUREMENT BY MEANS OF THE HYDROGEN CLEARANCE METHOD

A polyethylene tube was attached to the tracheal cannula of anesthetized animals to allow the addition of hydrogen gas to the inspired air. Once the S1 area was localized, following the procedure stated above, a ring (3.5 mm internal diameter and 2 mm height) was positioned over the area by means of a micromanipulator, without pressing the pial vessels on the cortical surface. The rest of the cortex not covered by the ring was sealed with Vaseline. The ring was provided with inlet and outlet tubing connected to syringes to allow renewal of solutions.

Recording of brain potentials and hydrogen current was performed by means of a platinized platinum wire, 30 μ in diameter, insulated with varnish except 500 μm adjacent to the tip. The electrode, driven by a micromanipulator, was inserted within the cortex at the site of minimum latency S1 response until the tip was 1 mm below the surface, in order to constantly record blood flow from the same functional area of cortex. To ascertain the effect of the evoked potential on cortical blood flow, in half of the experiments the stimulator was switched off as soon as the S1 area was localized and was kept off during the rest of the experiment. In the other half the stimulator remained on during the whole experiment. The somatosensory response was constantly reversed in polarity after insertion at this depth.

The reference electrode was a loop of Ag-AgCl wire at the subcutaneous space in the neck. The electrodes were fed to a Keithley 414A Picoammeter, which output led to a DC channel of a Nihon-Kohden polygraph to record permanently the ECG and the change in hydrogen current. To record the evoked response...
potentials, the output from the Picoammeter was fed to a Tektronix 502 cathode ray oscilloscope triggered by the stimulator to which a Nihon-Kohden kimographic camera was attached (fig. 1).

As the record was somewhat irregular shortly after insertion, the preparation was left two hours to stabilize before taking measurements. Local blood flow was determined from the tissue hydrogen desaturation slope according to Auckland et al. To accomplish this, hydrogen was admitted to the tracheal cannula and the gas flow was maintained constant until the current from the tissue electrode showed a steady state. Hydrogen flow to the tracheal cannula was then stopped and the desaturation curve recorded. The half time for washout was determined from the record, and local blood flow was calculated from the equation \( k = 0.693/t_{1/2} \), where \( k \) has the dimension ml/ml/min. Values of blood flow were finally expressed as ml/100 gm tissue/min. Determinations were performed at a rate of one every 15 to 20 minutes.

Hydrogen desaturation curves, in the conditions stated above, were always monoexponential.

The \( \text{H}_2 \) inflow to the tracheal cannula was adjusted to the minimum compatible with a change in tissue hydrogen concentration satisfactory with respect to detection of the curves.

Drugs were dissolved in artificial CSF\(^2\) and the fluid was introduced into the ring. The drugs used were: atropine sulphate (Purest), acetylcholine hydrochloride (Roche), eserine sulphate (Sandoz), carbaminoxycholine chloride (Merck, Sharp & Dohme), pilocarpine nitrate (Merck), arecoline bromhydrate (Nutritional Biochemicals Corporation), gamma-aminobutyric-acid (Sigma), and magnesium sulphate (Anedra).

**Results**

**STAGES OF URETHAN ANESTHESIA**

The recording of electrocorticogram (ECG), electrical activity of dorsal hippocampus (HIP), electromyogram of neck muscles (EMG), and respiratory frequency (pneumogram) allowed us to detect three stages (1, 2 and 3) characterized by a certain combination of the mentioned variables that are depicted in figure 2. Stage 1 was present only at the beginning of the experiment or after an overdose of urethan; its duration was related to the dose used and ranged from 40 minutes (1.2 gm/kg) to 305 minutes (1.8 gm/kg). Stage 2 followed stage 1 with a gradual transition but stage 3 and stage 2 alternated with net transitions. In a series of experiments at a dose of 1.5 gm/kg, the duration of each second or
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TABLE 1
Blood Pressure, Heart Rate, Respiratory Frequency and Arterial Pco2 in Stages 2 and 3 of Urethan Anesthesia

<table>
<thead>
<tr>
<th></th>
<th>Blood pressure (mm Hg)</th>
<th>Heart rate (beats/min)</th>
<th>Respiratory freq (breaths/min)</th>
<th>Arterial Pco2 (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 2</td>
<td>78.6 ± 0.81</td>
<td>359.1 ± 2.84</td>
<td>93.4 ± 0.48</td>
<td>42.5 ± 5.1</td>
</tr>
<tr>
<td>Stage 3</td>
<td>77.9 ± 0.54</td>
<td>342.4 ± 2.50</td>
<td>122.2 ± 1.40</td>
<td>26.2 ± 2.2</td>
</tr>
</tbody>
</table>

Mean ± SE. Determinations were performed in groups of ten animals.

third episode was very variable (range 10 seconds to 40 minutes). However, when the total time spent in each stage was computed for every experiment, it was found that the time spent in stage 3 was 60% ± 5.9 (mean ± SE, n = 16) of total time. Blood pressure and cardiac frequency had not changed between stages 2 and 3, but arterial Pco2 was somewhat lower in stage 3, presumably as a consequence of an improvement in alveolar ventilation (table 1). The somatosensory evoked potential recorded on the surface was constantly positive-negative during stage 1 (except when it coincided with a burst of cortical activity). During stage 2 it was very variable and during stage 3 an early negativity appeared and the shape of the wave was again very constant. Peak latency of the negative wave recorded by the depth electrode was shorter in stage 3 (13.82 msec ± 0.32) than in stage 1 (14.75 msec ± 0.14) or 2 (14.78 msec ± 0.11).

MEASUREMENT OF CORTICAL BLOOD FLOW IN THE DIFFERENT STAGES OF URETHAN ANESTHESIA
Stage I was not studied because it occurred mostly during the stabilization period. The comparison of blood flow values obtained during stages 2 and 3 showed a significant difference, both in experiments with or without S1 evoked potentials (fig. 3). The presence of a somatosensory potential significantly increased blood flow in both stages. As there were no differences in the values of mean blood pressure recorded in stages 2 and 3 (table 1) or with and without stimulation, it is considered that the reported changes in cortical blood flow reflect changes in cortical vascular resistance. The fact that arterial Pco2 was not higher in stage 3 rules out a possible effect through changes in this variable.

EFFECT OF TOPICAL DRUGS ON CORTICAL BLOOD FLOW
Cholinergic substances added in different experiments to the fluid bathing the area of cortex under study significantly increased blood flow (table 2). To disclose if the cholinergic vasodilatation could be due to activation of nerve cells by cholinergic drugs or to a direct action on smooth muscle cholinergic receptors, experiments were performed in which electrocortical activity was blocked by topical application of 0.1 M MgSO4 or 0.2 M gamma-aminobutyric-acid (GABA), according to Bindman et al.21

When abolition of unitary and mass evoked activity was complete, cholinergic drugs were added in a solution also containing 0.1 M MgSO4 or 0.2 M GABA. In those conditions, pilocarpine (0.5 mg/ml) increased CBF by 54.5% above control values (p < 0.001, N: 5) in MgSO4 pretreated cortex. Arecoline increased CBF by 47.5% above control values (p < 0.001, N: 7) in GABA pretreated cortex. Despite the fact that MgSO4 or GABA abolished electrical activity, these drugs did not significantly modify CBF with respect to the values prior to their addition. Acetylcholine (10 μg/ml) and eserine (200 μg/ml), carbachol (100 μg/ml) and atropine (400 μg/ml) did not change pH of artificial CSF, but the rest of the drugs made CSF...
more acidic than control CSF by a value never
greater than 0.4 pH unit. However, observed
changes cannot be related to this fact as control
experiments, in which CSF pH was lowered by 0.6
pH unit, did not show changes in cortical blood flow
(table 2).

As can be seen in figure 4, pilocarpine
considerably decreased the evoked activity although
increasing blood flow (steeper H₂ washout slope).
The addition of atropine counteracted the effect of
pilocarpine on blood flow (H₂ slope returned to its
original level), but it greatly increased evoked
activity. In view of these facts, it was decided to test

the effect of drugs affecting cholinergic transmission
on the spontaneous increase in blood flow of cortical
desynchronization (stage 3). It was found that in
stage 2 the values of blood flow after local atropine
did not differ from the control determinations, while
an increase followed local eserine. In stage 3, local
atropine diminished cortical blood flow to the level
of control stage 2, and local eserine raised cortical
blood flow by about 100% of the control value
(table 3).

Figure 5 shows the difference in the effect of
serine depending on the electrical state of the cortex
in the course of a typical experiment. Despite the

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**TABLE 2**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. in ring fluid (µg/ml)</th>
<th>Cortical blood flow (ml/100 gm/min)</th>
<th>Increase (X ± SE)</th>
<th>P*</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol</td>
<td>100</td>
<td>64.7</td>
<td>31.3 ± 5.9</td>
<td>&lt; 0.01</td>
<td>5</td>
</tr>
<tr>
<td>Arecoline</td>
<td>500</td>
<td>53.4</td>
<td>51.0 ± 6.7</td>
<td>&lt; 0.0025</td>
<td>5</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>10</td>
<td>34.8</td>
<td>51.7 ± 17.8</td>
<td>&lt; 0.05</td>
<td>7</td>
</tr>
<tr>
<td>Eserine</td>
<td>25</td>
<td>48.2</td>
<td>2.3 ± 10.7</td>
<td>&lt; 0.01</td>
<td>5</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>200</td>
<td>57.0</td>
<td>2.3 ± 10.7</td>
<td>&lt; 0.01</td>
<td>5</td>
</tr>
<tr>
<td>Eserine</td>
<td>500</td>
<td>77.0</td>
<td>52.2 ± 10.7</td>
<td>&lt; 0.01</td>
<td>5</td>
</tr>
<tr>
<td>Acidified CSF</td>
<td>(0.6 pH unit)</td>
<td>31.9</td>
<td>2.3 ± 2</td>
<td>NS</td>
<td>5</td>
</tr>
</tbody>
</table>

*Student's "t" test for paired samples.
CSF pH was lowered by addition of HCl.

---

*FIGURE 4*

Record of hydrogen clearance curves and S₁ evoked potential from the same electrode within
cerebral cortex. Negative upward. The recorder was stopped during the saturation period.
Curves were taken at 15-minute periods. At first arrow a solution of pilocarpine (500 µg/ml)
was topically applied to the cortex. At second arrow it was replaced by a solution containing
500 µg/ml pilocarpine and 400 µg/ml atropine. Time calibration: 40 seconds (explanation in
the text).
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**TABLE 3**

Cortical Blood Flow After Topical Application of Atropine or Eserine in Stages 2 and 3 of Urethan Anesthesia

<table>
<thead>
<tr>
<th>Stage</th>
<th>Experimental condition</th>
<th>Cortical blood flow (ml/100 gm/min)</th>
<th>n*</th>
<th>p versus control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 2</td>
<td>Control</td>
<td>52.1 ± 2.58</td>
<td>36/14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Atropine (400 µg/ml)</td>
<td>56.6 ± 6.40</td>
<td>15/10</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>Eserine (500 µg/ml)</td>
<td>77.6 ± 7.96</td>
<td>21/11</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Control</td>
<td>76.6 ± 3.11</td>
<td>22/11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Atropine (400 µg/ml)</td>
<td>48.92 ± 4.06</td>
<td>25/7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Eserine (500 µg/ml)</td>
<td>140.9 ± 2.75</td>
<td>21/10</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Mean ± S.E.

*No. of determinations/no. of animals.

†p of the difference versus control.

Fact that eserine was present in the ring during the period demarcated by the full line, the great increase in blood flow observed after local eserine application ended as soon as the cortex changed from stage 3 to stage 2.

**Discussion**

The urethan anesthesia permits operation on the exposed cortex of an immobilized animal, spontaneously fluctuating between a synchronized and a desynchronized state. Lincoln considers the oscillation between stages 2 and 3 of urethan anesthesia as analogous to the sleep-wakefulness cycle; an assumption supported on the appearance of a stage resembling REM sleep in his experiments, although he judged it only from the electrocortical record. In our experiments, however, we could not confirm that assumption since we never found a state with cortical desynchronization, hippocampal theta rhythm and relaxation of neck muscles that could be ascribed to an REM sleep episode.

Nevertheless, stage 3 very much resembled waking (cortical desynchronization with hippocampal theta rhythm, activity in neck muscles and opening of the eyes). In addition, a stage 3 episode could be triggered by a painful stimulus or a sound. Haining et al. have measured blood flow in the frontal cortex of unanesthetized rats, also using the hydrogen clearance method, and the result they obtained, 79.6 ml/100 gm/min (average of five rats), was quantitatively similar to the value of 76.6 ml/100 gm/min that we obtained in stage 3 unstimulated cortex (table 3). This coincidence gives further support to the idea that the stage 3 of urethan anesthesia is a stage resembling waking. The hydrogen clearance method has the great advantages that it gives a quantitative value in absolute units, and that the measurement can be localized to the region that surrounds the electrode. The inhalation method of saturating the tissue with hydrogen, used in the present experiments, is liable to a certain inaccuracy with respect to the saturation by means of an intra-arterial bolus of hydrogen-saturated saline as reported by Meyer et al. Nevertheless, even if the error existed, it has been constant throughout all the experiments.

The increase in blood flow that follows cortical arousal currently has been ascribed to the local vasodilating action of CO₂ or other products of increased metabolism which accompany neuronal activation. Nevertheless, our results, although they do not discard the existence of metabolic factors in the control of cortical blood flow, seem to support the existence of a neurogenic control cholinergically mediated, since the increase in blood flow that accompanies spontaneous cortical desynchronization can be prevented by local atropine, and is exaggerated by local eserine, and cholinergic drugs...
increase blood flow when topically applied. The possibility that the above-mentioned drugs could have acted indirectly through metabolic activation or inhibition of nerve cells cannot be discarded, since metabolism was not directly evaluated in the present experiments. However, the changes in cortical electrical activity induced by these drugs make this unlikely. Atropine, although preventing the vasodilatation of stage 3, increased $S_1$ evoked activity in accordance with previous reports.

Cholinomimetic drugs decreased the level of $S_1$ evoked activity, also in line with previous results, but greatly increased blood flow, an effect that persists even after complete block of electrocortical activity by GABA or MgSO$_4$. The increase in cortical blood flow found with cholinergic drugs is in line with numerous previous reports.

Furthermore, as has been shown by others, acetylcholine is released from the cerebral cortex during desynchronization, a fact suggesting that there are, within the tissue, presynaptic fibers releasing acetylcholine at the moment of desynchronization that could be related to the mechanism discussed above.

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