In Vivo Evidence That an Adenylate Cyclase-cAMP System Dilates Cerebral Arterioles in Mice

William I. Rosenblum, MD

Pial arterioles of living mice anesthetized with urethane were monitored by television microscopy. I tested the existence of an adenylate cyclase-cyclic adenosine monophosphate (cAMP) system for dilating the arterioles by topically applying the following drugs: cAMP (10^{-3} M), its more potent analogue dibutyryl cAMP (10^{-3} and 10^{-4} M), and forskolin (10^{-5} M). Forskolin activates endogenous adenyl cyclase, which leads to increases in endogenous cAMP. Each drug was applied for 30 seconds; all three produced dilation. I then applied either cAMP or forskolin in the presence or absence of 10^{-4} M isobutylmethylxanthine (IMX), an inhibitor of endogenous phosphodiesterase, which destroys cAMP. The presence of IMX significantly potentiated the dilation produced by exogenous cAMP and forskolin. These data indicate that cerebral surface arterioles of mice respond to cAMP with dilation and contain the enzymes for producing and inactivating this dilator. The existence of an adenylate cyclase-cAMP dilating mechanism in pial arterioles does not rule out the simultaneous existence of other dilating mechanisms. (Stroke 1988;19:888–891)

Among the several metabolic pathways implicated in control of vasodilation, one pathway involves activation of adenylate cyclase with production of cyclic adenosine monophosphate (cAMP) and another involves activation of guanylate cyclase with production of cyclic guanosine monophosphate (cGMP). Existence of the adenylate cyclase pathway has been supported by in vitro studies of large blood vessels including those supplying the brain. In addition, there is a study in which an activator of adenylate cyclase given intravenously increased cerebral blood flow; the precise site of vasodilation was not identified. So far as I know there are no other in vivo studies supporting a role for the adenylate cyclase pathway and, particularly, no in vivo microvascular studies concerning either the brain or any other organ.

My investigation examines dilation in situ, in vivo in the cerebral microcirculation of mice and demonstrates the existence of a vasodilating adenylate cyclase pathway by showing 1) that cAMP, the product of this pathway, dilates the arterioles; 2) that an activator of adenylate cyclase dilates the arterioles; and 3) that an inhibitor of the endogenous enzyme that destroys cAMP enhances the dilating activity of cAMP and enhances the dilating activity of the activator of adenylate cyclase.

Materials and Methods

Mouse preparation. Male mice (ICR strain, Dominion Labs, Dublin, Virginia) were anesthetized with urethane, and the pial arterioles were exposed by craniotomy as described. The body temperature was maintained at 37° C. The cerebral surface was continuously suffused at 2 ml/min with artificial cerebrospinal fluid (CSF) at 37° C and pH 7.35-7. The precise site of vasodilation was not identified. In each mouse one arteriole 30–50 μm in diameter was arbitrarily selected for observation and, particularly, no in vivo microvascular studies concerning either the brain or any other organ.

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Drugs used. Drugs were administered topically in a 1-ml bolus over 30 seconds at the same rate as and in place of the suffusate of artificial CSF. The bolus consisted of the drug dissolved in artificial CSF at 37° C and pH 7.35. The pH of each bolus was checked before administration. All drugs were ob-

From the Department of Pathology (Neuropathology), Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia.

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Address for reprints: William I. Rosenblum, MD, Medical College of Virginia, Box 17, MCV Station, Richmond, VA 23298.

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tained from Sigma Chemical Co., St. Louis, Missouri. The drugs were isobutylmethylxanthine (IMX), an inhibitor of cAMP phosphodiesterase; forskolin, an activator of adenylate cyclase; and cAMP sodium salt; and its analogue, dibutyryl cAMP. All the drugs except forskolin were soluble in water; stock solutions of forskolin were made in ethanol. Final dilution in artificial CSF gave a concentration of ethanol that was only 0.01%. Preliminary studies showed that a 1-ml, 30-second bolus of this concentration of ethanol had no effect on arteriole diameter.

Experimental design. Several experiments were performed, each with 10 mice. To 10 mice I applied $10^{-3}$ M cAMP, a high dose selected because it was indicated by the literature. In 10 different mice I applied $10^{-3}$ and $10^{-4}$ M dibutyryl cAMP at 15-minute intervals, with washout by the suffusate between the two applications. The diameter of the arteriole returned to baseline between applications, and the two responses were compared using the nonparametric Wilcoxon signed ranks test for matched pairs rather than the paired $t$ test because data expressed as percentages is not normally distributed and, hence, parametric statistics are not appropriate. In 10 different mice I determined the response to $10^{-6}$ M forskolin. In two more studies, each using 10 mice, I tested the response to $10^{-3}$ M cAMP or $10^{-4}$ M forskolin in the absence and presence of $10^{-4}$ M IMX. Fifteen minutes elapsed between the two administrations, during which time the suffusate washed the drugs out and restored arteriole diameter to control levels. The order of administration (i.e., drug alone or drug + IMX) was randomized. The two responses were compared using the Wilcoxon test. Fifteen minutes of washout followed the second administration, and then the response to IMX alone was tested.

Results

Dilation by cAMP and its analogue. When cAMP was applied at $10^{-3}$ M, 10 arterioles with a (mean ± SD) diameter of $40 ± 5$ μm were relaxed by $6 ± 2%$ (mean ± SD). Every arteriole dilated. When an equimolar concentration of dibutyryl cAMP was used, 10 arterioles with a mean ± SD diameter of $40 ± 2$ μm were relaxed by $14 ± 5%$, while a one-tenth concentration produced a dilation of $8 ± 3%$ in the same vessels ($p<0.01$, Wilcoxon test). Again, every arteriole dilated. As I note below, further studies with this dose of cAMP likewise produced dilation in every case. Thus the dilation, though modest, was not due to random or chance variations in vessel size. Such variations would be expected, at best, to produce dilations only half the time, with constrictions the other half. A Fisher test, comparing the observed with the predicted chance occurrence of dilation, showed that dilations occurring after application of $10^{-3}$ M cAMP were not explainable by chance ($p<0.01$).

Table 1. Potentiation of cAMP Dilation of Pial Arterioles in Mice by Phosphodiesterase Inhibitor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diameter (μm)</th>
<th>% change in diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP $10^{-3}$ M</td>
<td>38 ± 5</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>cAMP $10^{-3}$ M + IMX $10^{-4}$ M</td>
<td>38 ± 5</td>
<td>12 ± 2*</td>
</tr>
<tr>
<td>IMX $10^{-4}$ M</td>
<td>38 ± 5</td>
<td>-3 ± 1</td>
</tr>
</tbody>
</table>

Values are mean ± SD for 10 mice. IMX, isobutylmethylxanthine.

*Significantly greater than response to cAMP alone, $p<0.01$.

Dilation by activation of adenylate cyclase. Forskolin, an activator of adenylate cyclase, produced dilation. Dilation by cAMP and its analogue, at concentrations to which the vessels were exposed, was always greater in magnitude than that caused by forskolin. Table 1 shows the potentiating effect of IMX. The mean ± SD relaxation of $8 ± 2%$ to cAMP alone was increased by half, to $12 ± 3%$ ($p<0.01$, Wilcoxon test). The effects of IMX alone were also tested, and a slight constriction of the vessels was noted ($3 ± 1%$ of resting diameter). Thus, the significant potentiating action of IMX was not due to additive dilation effects produced by IMX and cAMP alone. As shown in Table 2, IMX also potentiated the dilation produced by forskolin. In 10 different mice forskolin produced a mean ± SD dilation of $10 ± 2%$, which was increased to $15 ± 2%$ by the simultaneous application of $10^{-4}$ IMX ($p<0.01$, Wilcoxon test). In this series of mice, IMX by itself failed to alter diameter, mean ± SD diameter after IMX being $100 ± 3%$ of that before IMX.

Discussion

The important new data in my study indicates that in vivo, the arterioles on the cerebral surface of mice display mechanisms for controlling dilation that depend upon activation of adenylate cyclase. Forskolin, a well-known activator of endogenous adenylate cyclase, produced dilation. Dilation could be produced by the product of adenylate cyclase activity, cAMP, and by an analogue of cAMP. The analogue, dibutyryl cAMP, produced dilations 50% larger than those produced by equimolar cAMP. This analogue is known to be more potent than cAMP, because it penetrates intact cells more readily and/or because it resists breakdown by phosphodiesterase. Phosphodiesterase is the endogenous destroyer of cAMP. IMX, an inhibitor of phosphodiesterase, potentiated the dilating action of both exogenous cAMP and forskolin, the activator of adenylate cyclase that produces endogenous cAMP. Thus, the presence of an adenylate cyclase-cAMP-dependent relaxing mechanism was also supported by the actions of IMX.
TABLE 2. Potentiation of Forskolin Dilation of Pial Arterioles in Mice by Phosphodiesterase Inhibitor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diameter (μm)</th>
<th>% change in diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin 10^{-4} M</td>
<td>39 ± 3</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Forskolin 10^{-4} M + IMX 10^{-4} M</td>
<td>38 ± 3</td>
<td>15 ± 2*</td>
</tr>
<tr>
<td>IMX 10^{-4} M</td>
<td>39 ± 3</td>
<td>0 ± 3</td>
</tr>
</tbody>
</table>

Values are mean ± SD for 10 mice. IMX, isobutylmethylxanthine.

*Significantly greater than response to forskolin alone, p<0.01, Wilcoxon signed ranks test for matched pairs.

The responses to cAMP and to 10^{-4} M dibutyryl cAMP represented diameter increases of approximately 4-10%. These dilations are modest, but they occurred in every mouse tested, a result highly significantly different from that expected from random noise or random spontaneous changes in diameter. The latter would lead to dilations in only half the mice. If parametric statistical tests, like paired t tests, were applied to the data they would also show highly significant (p<0.01) differences from zero change, i.e., no response to the nucleotide. These changes reflect a mechanism able to respond to endogenous cAMP concentrations. Diameter changes of this magnitude can be physiologically significant because of the well-known exponential relation between diameter and flow. For example, a 6% change in diameter reduces the resistance of the affected segment by 19% if third-power relations are used and by 27% if a fourth-power relation is used. The dose of cAMP was high (10^{-3} M) as were the doses (10^{-3} and 10^{-4} M) of dibutyryl cAMP, but these doses cannot tell us the effective intracellular concentration of endogenously derived cAMP at the critical intracellular site. Thus, the doses I used in no way mitigate against the physiologic importance of the mechanism, the existence of which is supported by my data. Moreover, my experiments with forskolin provide the evidence that elevations of endogenous cAMP concentrations do indeed produce dilation of the same or greater magnitude than those produced by exogenous application of cAMP.

It is axiomatic that pharmacologic probes, no matter how specific, have actions other than those for which they were originally selected. One means of dealing with this problem is to use several drugs with the same purported specificity and to show that the result in question is obtained with each drug. Another approach is to use a variety of drugs, each of which interacts with a different point in the biochemical system under examination. I have elected the latter approach. Relaxation was produced by introducing exogenous cAMP or its analogue, a result suggesting a dilating mechanism sensitive to cAMP. Relaxation was produced by an activator of adenylate cyclase, a result suggesting the presence of intrinsic adenylate cyclase. Relaxation was potentiated in each instance by an inhibitor of phosphodiesterase, a result suggesting that the relaxations were indeed caused by a cyclic nucleotide and also suggesting the presence of an endogenous enzyme for getting rid of that nucleotide. Taken together, these data strongly support the conclusion that our probes did indeed work via machinery mediating the adenylate cyclase-cAMP system. I further believe that these probes worked directly on the pial arterioles.

I considered the possibility that suffusion of forskolin and IMX over the brain surface elevated cAMP levels within brain cells and that this led indirectly to vasodilation via activation of neurons and/or liberation from the brain of some other vasodilator. However, this hypothesis is highly unlikely for several reasons:

1. Suffusion of the pial surface with norepinephrine constricts pial arterioles rather than dilating them even though norepinephrine is a well-known elevator of neuronal cAMP concentrations. Thus, pial arterioles respond to the suffusate as they would even if totally removed from the underlying brain.

2. Acetylcholine and bradykinin are well-known elevators of cAMP concentrations in neurons, and acetylcholine is a well-known neurotransmitter. Suffusion of the pial surface with either agent does dilate pial arterioles, but this dilatation is mediated by action of the dilators on the arteriolar endothelium and is abolished by injuring this endothelium. Therefore, these dilators appear to act via mechanisms intrinsic to the vessels rather than to the underlying brain.

3. Brain cAMP concentrations are not elevated by a phosphodiesterase inhibitor in the CSF over the brain surface in the concentrations used here even after periods 30 times longer than the times used here.

All these facts taken together support my belief that the dilations produced or potentiated by forskolin or IMX in my study were due to actions on adenylate cyclase or phosphodiesterase within the walls of the pial arterioles rather than in the underlying brain.

My data does not rule out the presence of other dilating mechanisms, such as those mediated by the guanylate cyclase-cGMP system. Indeed, I have reported that mouse pial arterioles also have the dilating mechanism mediated by guanylate cyclase.

Others have demonstrated an accumulation of cAMP in isolated cerebral arteries of cats, paralleling relaxation by selected agonists. In isolated cerebral microvessels from rats and other species, some dilators have been shown to activate adenylate cyclase. However, the microvessels were largely capillaries rather than arterioles, and adenylate cyclase in capillaries is probably in the endothelium, where it may mediate permeability. This possibility was, for example, suggested in studies in which forskolin enhanced the accumulation of cAMP by adenosine analogues. Adenylate cyclase has been histochemically identified in endothelium of cerebral microvessels and in
the smooth muscle of such microvessels.\textsuperscript{17,31} The presence of adenylate cyclase in endothelium may have wider implications than control of permeability since in pial arterioles and in larger brain arteries some agonists produce dilation by causing endothelium to release one or more endothelium-dependent relaxing factors.\textsuperscript{4,24,25} My data do not rule out the possibility that endothelium, rather than muscle, was the site of cAMP action, in turn triggering production or release of a dilator.

My study appears to be the first in situ demonstration that the adenylate cyclase-cAMP system can act in vivo to reduce the tone of cerebral arterioles. One previous study showed increased cerebral blood flow after activation of endogenous adenylate cyclase by intravenous forskolin.\textsuperscript{5} The site or sites of adenylate cyclase activation cannot be determined in that kind of study. The pial arterioles comprise an important segment of cerebral vascular resistance.\textsuperscript{32} However, the physiologic or pathologic importance of the adenylate cyclase mechanism in these arterioles ultimately depends upon the capacity, in health and disease, of the humoral agents and neurotransmitters that reach these vessels to activate adenylate cyclase. The in vitro literature shows that a wide variety of putative relaxants can activate adenylate cyclase. This makes it more likely that the system is important in the control of tone.

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W I Rosenblum

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