Endothelium-Dependent Effects of Substance P and Calcitonin Gene-Related Peptide on Mouse Pial Arterioles

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Background and Purpose: The effects of substance P (SP) and calcitonin gene-related peptide (CGRP) were tested on pial arterioles of mice. This was done because (1) perivascular peptidergic nerves may play an important role in modulation of cerebrovascular responses; (2) there are conflicting data concerning the mechanism of action of CGRP; (3) there are few or no studies directly testing the endothelium dependence of dilation by these peptides in the cerebral circulation; and (4) we wished to extend previous observations of mice by comparing peptidergic responses in the mouse with those published for other species.

Methods: The pial arterioles were monitored in vivo using video microscopy and image-shearing techniques for measuring diameter. Focal endothelial injury was produced with a laser–Evans blue technique. Responses to SP and CGRP were tested before and after endothelial injury. They were also tested before and during treatment with agents that interfere with responses mediated by endothelium-deriv ed relaxing factor (EDRFAC). They were also tested before and during treatment with indomethacin.

Results: Both CGRP and SP produced dilation that was blocked by endothelial injury and by agents interfering with responses mediated by EDRFAC. Indomethacin had no effect.

Conclusions: SP and CGRP produce endothelium-dependent dilations. These dilations are probably mediated by EDRFAC. With respect to SP, these results are similar to those reported for other vessels and species. With respect to CGRP, the finding of endothelium dependence has not been previously reported for cerebral vessels. However, very few species have been tested. Reports of other vascular beds in other species sometimes parallel and sometimes contradict our findings with CGRP. (Stroke 1993;24:1043-1048)

Key Words • endothelium • vasodilation • mice

For many years we have used mouse pial arterioles as an in vivo model of the cerebral vasculature. Many of our observations are useful in understanding cerebrovascular control mechanisms in other species, for example, our demonstration of endothelium-dependent vasodilation.12 The following studies of peptide action on these vessels was undertaken for two reasons. First, we wished to see if mouse pial arterioles reacted to calcitonin gene-related peptide (CGRP) and substance P (SP) as do cerebral vascular beds in other species. Such a similarity would complement previously demonstrated similarities between mouse pial vessels and the cerebral vessels of other species. Second, we wished to study these particular peptides because of the recent important findings concerning the possible relation of these vasodilators to cerebrovascular control.3-12 These peptides are contained within nerves innervating the cerebral blood vessels.3 Recently, some peptides have been shown to play a role in mediating the

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vasodilation that occurs in experimental models of ischemia,8-10 hypertension,6,7 and seizures.7 In addition, a neuropeptide may mediate the vasodilation produced by local application of nitrosodilators to cat pial vessels.11 The studies reported below include direct testing of the endothelium-dependent action of CGRP and SP and also utilize inhibitors of dilations mediated by the endothelium-derived relaxing factor for acetylcholine (EDRFAC), a mediator of undetermined chemical composition in cerebrovascular endothelium.13 The studies of endothelium dependence and its relation to EDRFAC are of interest in view of conflicting data concerning several different vascular beds and the endothelium dependence of CGRP in particular.3,14-20

Methods

The methods have been described in detail many times.2,21-23 Briefly, male mice, ICR strain (Harlan Sprague Dawley, Indianapolis, Ind), were anesthetized with urethane. A tracheostomy and craniotomy were performed. The dura was stripped, exposing the transparent arachnoid and the underlying vessels on the brain surface (pial vessels). The mice were maintained at 37°C. The craniotomy site was continuously suffused with Elliott's solution24 at pH 7.3 to 7.4. All drugs were given in this solution at a final pH that was the same as

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the pH of the regular suffusate. Diameters were monitored via a video microscope and an image-splitting device. Image-splitting techniques permit measurements of less than 0.5 μm.25,26 Only one arteriole was used per mouse. The arteriole was arbitrarily selected from those 25 to 45 μm in internal diameter. Changes in diameter were expressed as a percentage of baseline. When a drug was applied as a bolus, we determined the maximal change in diameter produced by that drug and used this as the response to the drug.

To injure the endothelium, a laser-dye technique was used27-29; injected intravenously Evans blue dye acts as an energy-absorbing, heat-generating target; the HeNe laser beam, 36 μm in diameter, is focused through the objective lens of the microscope. We have published many studies that used this technique to demonstrate endothelium-dependent responses.2,23,27-29 We have shown that dilations by sodium nitroprusside and by a cyclic adenosine monophosphate analogue are not affected by the injury.2,30 The latter are well-known endothelium-independent relaxing agents, hence the lack of inhibition by the laser-dye technique supports the contention that only the endothelium is injured and not the smooth muscle.

Drugs Used

The following drugs were obtained from Sigma, St Louis, Mo: N⁴-monomethyl-L or D-arginine acetate (L-NMMA or D-NMMA); N⁴-nitro-L-arginine (NNA); and SP 2-chloroadenosine. Human CGRP was obtained from Bachem-California, Torrance, Calif. Sodium indomethacin trihydrate was a gift from Merck, Rahway, NJ. Prostacyclin was obtained from Cayman, Ann Arbor, Mich. Drugs were dissolved in deionized water, and further dilutions were made just before use in Elliott's solution. There was never more than 10 μL of water added to each milliliter of Elliott's solution. The final pH was always that of Elliott's solution alone (7.3 to 7.4). The stock solutions were made each day and kept on ice. The pH of the stock solution was 7.4. Responses late in the day did not differ from those early in the day, indicating no breakdown during the day in the stock solution.

Experimental Design

Each mouse underwent a 30-minute period of equilibration with the Elliott's solution. CGRP was applied as a 1-mL bolus over a period of 1 minute. Substance P was applied as a 3-mL bolus over a 3-minute period. Successive doses were 15 minutes apart. When the laser-dye combination was used, the dye was injected at the beginning of the 30-minute equilibration period. The dilating drug was then tested. Five minutes later, the laser was permitted to hit the vessel. Ten minutes later, a second application of the dilator was made. Fifteen minutes later, a third application was made at a site 100 μm from the site of injury. This last application served as a "time control" to show no deterioration of the preparation over time.

When L-NMMA, D-NMMA, or NNA was used, only one of these drugs was tested per study. L-NMMA and NNA were used because they prevent dilation mediated by EDRAch. This may be because they inhibit the enzyme synthesizing EDRAch.31,32 However, in this preparation they appear to work by generating an oxygen-centered free radical via an incompletely defined pathway.33 The radical destroys EDRAch.34,35 D-NMMA was also used in our investigation as a control because it is reported to be the inactive enantiomer of L-NMMA.31 The suffusion of these drugs began 10 minutes before application of the peptide, and they were also present in the solution containing the peptide. In each study, five mice were first tested with dilator alone followed by peptide plus drug. The sequence was reversed in five additional mice. Sequence had no effect on outcome, so the two groups were combined, giving n=10.

Indomethacin was tested in the manner described for L-NMMA. The high dose used (40 μg/mL) was selected because it was required to block constriction by 10 μg/mL sodium arachidonate in these mice.

Statistics

Since each arteriole served as its own control and the data were expressed as a percent change in diameter, the responses before and after treatment were compared using the Wilcoxon matched pairs test.36 A treatment effect was considered significant when the null hypothesis was rejected at the .05 level. Although the data were not analyzed using a comparison of means, the means and standard deviations are always given as a convenient way of presenting the results.

Results

CGRP relaxed vessels in a dose-dependent fashion. When 10⁻², 10⁻¹, and 10⁻⁰ mol/L doses were given to one set of mice (n=4), the diameter (39±2 μm) increased 5±1%, 6±2%, and 13±5% (mean±SD), respectively. Responses differed moderately in magnitude in different sets of mice, but whenever it was tested, a dose response was always observed. The reason for different magnitudes of response in different groups could not be explained.

SP 10⁻⁶ to 10⁻⁴ mol/L also dilated pial arterioles, but a definite dose response was not found. The reasons for this were unclear. Tachyphylaxis sometimes appeared with diminution but not loss of response to a second dose given 15 minutes after the first. However, no tachyphylaxis was noted to SP in studies of the action of D-NMMA, and there was no influence of treatment sequence on the results of the study in which SP was given twice, once with and once without L-NMMA.

Both the dilations by CGRP and by SP were markedly inhibited by endothelial injury. This is shown in Table 1. For CGRP, in 4 of the 10 mice, the response to CGRP was completely blocked by the injury. In all 10 mice, the response to CGRP was intact 100 μm away from the injury, showing that the diminished response at the injured site was not caused by deterioration of the preparation over time. In fact, the mean increase in diameter at the distant site was 7±2%, identical to the original preinjury response in the adjacent arteriolar segment. In the case of SP, endothelial injury totally abolished the response in 9 of 10 mice. Again, a third test of the peptide, 15 minutes later and 100 μm away, showed a full response to SP. Consequently, the loss of response at the injured site cannot be due to tachyphylaxis or to fatigue of the preparation.

The evidence that the laser-dye technique injures only endothelium and not vascular smooth muscle2,30
was further supported by the failure of laser/dye to inhibit prostaglandin I\(_2\) (PGI\(_2\)) in the present study (Table 1). The laser-dye combination also failed to inhibit dilation by 2-chloroadenosine. Here, two groups were compared rather than using each mouse as its own control. Each group (n=10) received two cumulative doses of 2-chloroadenosine, 10\(^{-4}\) mol/L and 5\times10\(^{-4}\) mol/L. Dilations were 6\(\pm\)2% and 11\(\pm\)3% in the controls. Identical responses (6\(\pm\)3% and 11\(\pm\)5%) were observed in the laser-dye group.

In the studies of the action of L-NMMA or NNA on CGRP or SP, we first selected concentrations of the arginine analogues that we knew had little or no effect on diameter. This was confirmed by the present results. In all except one study, at least half the vessels showed no change in diameter after 10-minute suffusion by arginine analogue, whereas constriction was seen in the remaining vessels. The effects of L-NMMA or NNA on dilation by peptide were the same whether the vessel was or was not constricted by the arginine analogue. CGRP was inhibited by both L-NMMA 10\(^{-6}\) and NNA 10\(^{-5}\) (Table 2). We previously showed that 10\(^{-6}\) mol/L L-NMMA blocked dilation by acetylcholine (ACh),\(^23\) New data (not presented in the table) showed, as expected, that 10\(^{-5}\) mol/L NNA also blocked ACh (10\(^{-7}\) mol/L). Dilation to ACh was 4\(\pm\)2% before and 0\(\pm\)1% after 10 minutes of 10\(^{-5}\) mol/L NNA. Because 10\(^{-5}\) did not completely block CGRP, we used a higher dose of NNA. This caused an 8% to 14% constriction in three mice, with little or no constriction (3% to 0%) in seven others. Overall, 10\(^{-4}\) mol/L NNA inhibited CGRP (Table 2), but the inhibition was not much greater than that produced by 10\(^{-5}\) mol/L NNA (Table 2). NNA 10\(^{-4}\) mol/L did not inhibit dilation by 10\(^{-7}\) mol/L sodium nitroprusside (5\(\pm\)1% versus 5\(\pm\)2% with and without NNA). We also tested D-NMMA, the enantiomeric isomer of L-NMMA. The D-NMMA had no effect against even a very low dose of CGRP, 10\(^{-8}\) mol/L. Vessels 39\(\pm\)1\% wide (n=5) dilated 4\(\pm\)1% to the CGRP both before and after 10 minutes of D-NMMA 10\(^{-6}\) mol/L.

SP was only slightly inhibited by L-NMMA 10\(^{-6}\) mol/L but was totally blocked by 10\(^{-4}\) L-NMMA: SP indeed produced a slight constriction (Table 2). The 10\(^{-4}\) dose of L-NMMA caused slight constriction in half the mice, but the block of SP occurred irrespective of the constriction. It made no difference whether SP was first tested in the absence of L-NMMA treatment or whether the L-NMMA-treated response occurred first and was followed by washout of L-NMMA and retest of SP 15 minutes later. D-NMMA 10\(^{-4}\) mol/L (Table 2) had no effect.

Because both CGRP and SP were endothelium dependent, we wished to see if their action was dependent on an endothelium-derived mediator other than EDRF\(_{AC}\). Prostacyclin was an obvious candidate. Therefore, we attempted to inhibit SP with the inhibitor of cyclooxygenase, indomethacin. However, 40 \(\mu\)g/mL of indomethacin failed to inhibit the dilation by either CGRP or SP. In the case of SP, 10\(^{-4}\) dilated vessels 7\(\pm\)2% before and 7\(\pm\)2% during indomethacin (40 \(\mu\)g/mL) treatment (n=6). The indomethacin itself failed to alter diameter. In the case of CGRP, 10\(^{-4}\) mol/L dilated vessels 4\(\pm\)1% before and 5\(\pm\)1% during indomethacin treatment.

**Discussion**

These data show that in mice, dilation by both CGRP and SP is endothelium dependent. This conclusion is based on observations before and after endothelial injury rather than merely on the presence or absence of inhibition by an inhibitor of EDRF\(_{AC}\) synthesis. Studies in the literature often use such inhibitors to examine the endothelium dependence of a response. Inhibition by

### Table 1. Effects of Endothelial Injury

<table>
<thead>
<tr>
<th>Dilator</th>
<th>Diameter ((\mu)m)</th>
<th>n</th>
<th>Before injury</th>
<th>After injury</th>
<th>100 (\mu)m away</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP (10(^{-8}) mol/L)</td>
<td>38(\pm)3</td>
<td>10</td>
<td>7(\pm)2</td>
<td>2(\pm)5*</td>
<td>7(\pm)2</td>
</tr>
<tr>
<td>SP (10(^{-4}) mol/L)</td>
<td>39(\pm)1</td>
<td>10</td>
<td>5(\pm)2</td>
<td>0(\pm)0*</td>
<td>6(\pm)2</td>
</tr>
<tr>
<td>PGI(_2) (2\times10(^{-7}) mol/L)</td>
<td>37(\pm)3</td>
<td>10</td>
<td>5(\pm)3</td>
<td>5(\pm)2</td>
<td>5(\pm)3</td>
</tr>
</tbody>
</table>

CGRP, calcitonin gene-related peptide; SP, substance P; PGI\(_2\), prostaglandin I\(_2\).

*P<.01 vs before injury, Wilcoxon test. All values are mean\(\pm\)SD.

### Table 2. Drug Effects on Dilation by Peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Diameter ((\mu)m)</th>
<th>n</th>
<th>Drug</th>
<th>% Increase in diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before drug</td>
<td>With drug</td>
</tr>
<tr>
<td>CGRP (10(^{-8}) mol/L)</td>
<td>37(\pm)3</td>
<td>10</td>
<td>L-NMMA (10(^{-4}) mol/L)</td>
<td>5(\pm)2</td>
</tr>
<tr>
<td></td>
<td>36(\pm)3</td>
<td>10</td>
<td>NNA (10(^{-5}))</td>
<td>5(\pm)3</td>
</tr>
<tr>
<td></td>
<td>35(\pm)4</td>
<td>10</td>
<td>NNA (10(^{-4}))</td>
<td>7(\pm)2</td>
</tr>
<tr>
<td></td>
<td>38(\pm)2</td>
<td>10</td>
<td>D-NMMA (10(^{-4}))</td>
<td>5(\pm)2</td>
</tr>
<tr>
<td></td>
<td>33(\pm)3</td>
<td>10</td>
<td>L-NMMA (10(^{-4}))</td>
<td>6(\pm)2</td>
</tr>
<tr>
<td></td>
<td>33(\pm)3</td>
<td>6</td>
<td>L-NMMA (10(^{-4}))</td>
<td>7(\pm)2</td>
</tr>
<tr>
<td></td>
<td>36(\pm)3</td>
<td>6</td>
<td>D-NMMA (10(^{-4}))</td>
<td>7(\pm)2</td>
</tr>
<tr>
<td></td>
<td>32(\pm)1</td>
<td>6</td>
<td>INDO (40 (\mu)g/mL)</td>
<td>7(\pm)2</td>
</tr>
</tbody>
</table>

CGRP, calcitonin gene-related peptide; SP, substance P; L-NMMA, N\(^6\)-monomethyl-L-arginine acetate; NNA, N\(^6\)-nitro-L-arginine; D-NMMA, N\(^6\)-monomethyl-d-arginine acetate; INDO, indomethacin.

*P<.01, tP<.05, Wilcoxon test.
such drugs can only imply involvement of EDRFACb. Such inhibition does not show conclusively that the endothelium is the source of the EDRFACb. The laser-dye technique used to test endothelium dependence did not interfere with either 2-chloroadenosine or PGI2. This supports previous studies showing that vascular smooth muscle is not injured.

We used L-NMMA, NNA, and indomethacin to help define the chemical mediator of the response to SP and CGRP. Indomethacin is a well-known inhibitor of prostaglandin synthesis. L-NMMA and NNA inhibit synthesis of EDRFACb or its precursor.13,31,32 However, when used in vivo in the mouse pial artery preparation, these arginine analogues may lead to superoxide formation and destruction of EDRFACb.33 In either case, inhibition of response by L-NMMA or NNA would signify that EDRFACb was the mediator of the response. The endothelium-dependent response to CGRP was markedly inhibited by 10^-6 M L-NMMA but not by D-NMMA. The response to CGRP was also inhibited by NNA, but even when a high concentration of NNA was used, the inhibition was less than by L-NMMA. Inhibition by both L-NMMA and NNA suggests that EDRFACb was the mediator of the response to CGRP, since L-NMMA and NNA are known to interfere with responses mediated by EDRFACb. Indeed, NNA inhibited dilation by ACh in the present study, and L-NMMA blocked dilation by ACh in a previous study.23 L-NMMA is a selective inhibitor of dilation in this preparation. That is to say, it is not an indiscriminate inhibitor of dilation. For example, it does not inhibit dilation by PGI2 or bradykinin.30 NNA was also selective; it was tested against and failed to interfere with sodium nitroprusside. We have no explanation for the fact that L-NMMA was a more potent inhibitor of CGRP than NNA.

The endothelium-dependent dilation by SP was only slightly impaired by low-dose L-NMMA but was blocked by high-dose (10^-6 mol/L) L-NMMA. The effect of the higher dose on SP was still stereospecific, since D-NMMA had no effect at that dose. The conventional interpretation of the SP data is that SP produces dilation via EDRFACb. We have no reason to offer another explanation. The responses to SP were not dose dependent. We have no definitive explanation for this. However, a slight constriction to SP was unmasked by endothelial damage. It may be that with increasing doses of SP, there are parallel increases of both dilating and constricting actions resulting in no net increase in the magnitude of the resultant dilation.

Neither CGRP nor SP were inhibited by a high dose of indomethacin. Thus, endothelium-derived prostanoids do not appear to be implicated as mediators of the dilation.

Our finding that SP is endothelium dependent is similar to virtually all reports of SP action both within and outside of the cerebral vasculature.3,5,14,31,33 However, our finding that CGRP is endothelium dependent contradicts other reports concerning brain blood vessels.14,15 and contradicts some reports concerning other vascular beds.14,20 We do not know if this represents a species difference, a difference in size of vessels studied, or a difference related to the in vitro nature of the latter studies14,20 as opposed to the in vivo technique used here. However, several other in vitro investigations of extracerebral vessels have concluded as we have that CGRP is endothelium dependent.16-19 In some of these cases, the action of CGRP has been inhibited by arginine analogues17,19 as in this study, thus implicating EDRFACb as the mediator. Some workers failed to find an elevation of cyclic guanosine monophosphate (cGMP) after CGRP treatment.18 They concluded from this that even though CGRP was endothelium dependent, EDRFACb could not be the mediator, since EDRFACb is thought to produce dilation via an increase of cGMP. However, others show that CGRP can indeed elevate cGMP in rodent aorta.16 Mouse pial vessels appear to fit into that group of vessels whose responses to CGRP are dependent on an endothelium-derived mediator resembling EDRFACb. However, this conclusion is largely based on complete inhibition of response by L-NMMA. Since even high concentrations of NNA only partially inhibited the response to CGRP, it is theoretically possible that L-NMMA might be acting by two mechanisms, one against EDRFACb, and one undefined. EDRFACb then would be one of two endothelium-derived mediators of CGRP action. The other mediator would also be L-NMMA sensitive. It would not be a product of cyclooxygenase activity because indomethacin had no effect on the action of CGRP.

In summary, our findings indicate that in mice as in other species, dilation of pial vessels by peptides can occur via an endothelium-dependent route. This is shown first by demonstrating loss of the response after endothelial damage in vivo. Such a demonstration appears to us to be more direct than the more common technique using a pharmacological inhibitor of some known endothelium-dependent pathway. When we used such pharmacological techniques, the data implicated EDRFACb as the mediator for both CGRP and SP. The demonstration of endothelium-dependent dilation by CGRP is at variance with other reports.3,15 However, we cannot find reports concerning CGRP, endothelium dependence, and pial vessels in species other than the cat. Therefore, no conclusions can be drawn with respect to whether the response of murine pial arterioles to CGRP is typical or atypical of mammalian pial vessels. Considered together, the demonstration of endothelium dependence, the known localization of CGRP and SP in perivascular nerves,3,6,10 and the effects of trigeminal ganglionecrosis or related procedures on cerebrovascular regulation10,12 support the assumption that peptides released by nerves can elicit vasodilation by traversing the vascular wall and reaching the endothelium, where they release EDRFACb.

Acknowledgment

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References

The accompanying article by Rosenblum et al raises at least two important issues regarding peptidergic mechanisms in cranial blood vessels. In a recent report, Wei, Boccocini, Kontos, and I described evidence supporting the formulation that calcitonin gene-related peptide (CGRP) release from sensory fibers mediates in part the vasodilating effects of two nitric oxide precursors, nitroglycerin and sodium nitroprusside. Hence, we observed that after chronic trigeminal nerve sectioning or after the application of a peptide CGRP antagonist to normal pial vessels, the ability of superfused nitroglycerin or sodium nitroprusside to relax cat pial blood vessels was significantly attenuated. As expected, relaxation by ATP or adeno-
sine was unaffected by either manipulation. Unpublished studies by us have since shown that CGRP(8-37), an antagonist, significantly attenuates vasodilation induced by nitric oxide itself. Although current wisdom places the receptors for CGRP on vascular smooth muscle cells, the findings of Rosenblum et al indicate that the endothelium and possibly nitric oxide may be critical for CGRP-induced relaxation of pial vessels (at least in the mouse). Hence, nitric oxide (or a related substance formed either from drugs or by vascular cells or neurons) releases CGRP from perivascular axonal stores, whereupon CGRP may promote endothelium-derived relaxing factor release from the endothelium. Not to be discounted, nitric oxide may also derive from
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