Effects of calcium antagonists on intracerebral penetrating arterioles in rats

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There is no direct information on the effect of calcium antagonists on intracerebral penetrating arterioles, which are responsible for a significant part of total cerebrovascular resistance. In a study on rats, the effects of four calcium antagonists (diltiazem, verapamil, nifedipine, and nimodipine) on isolated intracerebral penetrating arterioles with mean resting diameters (± standard error of the mean) of 52.3 ± 3.0 μm were investigated. Vessel diameters were monitored in vitro by means of a video microscope dimensional analyzer under constant transmural pressure (60 mm Hg) after cannulation. Each calcium antagonist produced maximal dilation of about 50% (diltiazem 46.4% ± 5.6%, verapamil 53.1% ± 6.0%, nifedipine 46.9% ± 6.1%, and nimodipine 47.1% ± 5.4%) with varied sensitivity (median effective dose (ED₅₀): diltiazem 1.52 × 10⁻⁶ M, verapamil 1.08 × 10⁻⁶ M, nifedipine 8.65 × 10⁻⁷ M, and nimodipine 1.62 × 10⁻⁹ M). Dilation effects persisted for a significantly longer time after washout with calcium antagonists such as diltiazem (15.5 ± 1.8 minutes), nifedipine (19.0 ± 3.9 minutes), and nimodipine (30.0 ± 1.6 minutes) than after washout with adenosine (8.5 ± 1.0 minutes). It appeared that the magnitude of vasodilation was greater and the duration of dilation after washout longer in intracerebral penetrating arterioles than that reported for pial arterioles, although sensitivity to each calcium antagonist was quite similar to that reported for larger cerebral arteries. These data provide a possible explanation for the apparent disparity between clinical efficacy and angiographically determined vessel diameter when patients with cerebral vasospasm are treated with calcium antagonists. These agents may have a greater effect on intracerebral penetrating arterioles than on angiographically visible larger arteries.

KEY WORDS · nimodipine · nifedipine · calcium antagonist · verapamil · diltiazem · microvasculature · rat

Calcium channel blockers cause vasodilation by preventing the influx of extracellular calcium into vascular smooth-muscle cells. Clinical and experimental studies indicate that calcium antagonists are effective in the treatment of cerebral ischemia after subarachnoid hemorrhage (SAH).¹,⁷,¹¹,²⁵

Much is known about the physiological contractile properties of cerebral arteries and pial surface arterioles. Extrapolation of these data to the level of the intracerebral microcirculation may not be justified. Longitudinal heterogeneity of responsiveness to vasoactive agonists in the cerebral circulation is well described, especially for adrenergic and serotonergic vasoconstrictors.¹⁰,¹² For example, norepinephrine, a potent constrictor of vascular smooth muscle in large arteries and arterioles in most peripheral vascular beds, has little effect on pial and intracerebral arterioles.¹²,²¹ Similarly, it may be inappropriate to assume that calcium antagonists act on intracerebral vessels in the same way that they act on larger cerebral arteries. The objective of the present study was to examine the effects of calcium antagonists on intracerebral arterioles.

Methods have been developed for the study of isolated intracerebral or penetrating arterioles.¹²-¹⁴ These techniques enable direct observations of the reactivity of viable penetrating arterioles while precisely controlling intra- and extraluminal fluid composition. In this paper, the potent and prolonged vasodilation effects of extraluminally applied calcium antagonists on the resting diameter of isolated intracerebral penetrating arterioles are reported.

Materials and Methods

Isolation and Cannulation of Vessels

Animal experimentation was conducted in conformity with the American Physiological Society's "Guiding Principles in the Care and Use of Animals." Methods
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for the isolation and cannulation techniques are described by Dacey, et al.12-14 Briefly, penetrating cerebral arterioles, 20 to 80 μm in diameter, were surgically isolated from the first (M1) portion of the middle cerebral artery of pentobarbital-anesthetized Sprague-Dawley rats weighing 300 to 400 gm. The vessel segments were transferred to a temperature-controlled chamber on the stage of a Nikon inverted microscope and were cannulated using glass pipettes. Vessel diameters and wall thickness were determined by means of a video-dimensional analysis system.

After cannulation, transmural pressure was applied at 60 mm Hg via the cannulating pipette and the external solution was brought from room temperature to between 37° and 38°C. Over an equilibration period of approximately 45 minutes, during which time the bath solution was changed three to four times, spontaneous tone developed. Responsiveness of the vessels was then assessed by changing the extraluminal pH from 7.3 to 6.8 and from 7.3 to 7.6.

Perfusion and Bath Solutions

The physiological salt solutions (PSS's) used in this preparation were modified Ringer's solutions with a composition as follows (in mM): NaCl 144, KCl 3.0, CaCl2 2.5, MgSO4 1.5, glucose 5, pyruvate 2, ethylenediaminetetra-acetic acid (EDTA) 0.02, 3-[N-morpholino] propanesulfonic acid 2.0 (MOPS), NaH2PO4 1.21, and bovine serum albumin 0.9 to 1 gm/100 ml. The intraluminal solution pH was maintained at 7.3 for all experiments. The extraluminal solution contained no albumin and the pH was varied from 6.8 to 7.6.

Dose-Response Curves of Calcium Antagonists

Control vessel diameter was defined as the diameter to which the vessels spontaneously contracted during the equilibration period in a bath solution of pH 7.3. Bath solutions of increasing concentrations of calcium antagonists were added sequentially to the cannulation chamber. The pH of each solution was measured prior to instillation into the chamber, and was found not to vary significantly from pH 7.3. The vessel diameter was allowed to stabilize over a period of 4 to 5 minutes between each change in bath solution. A control solvent-vehicle response experiment was carried out for nifedipine and nimodipine, which needed special solvent vehicles (ethanol and polyethylene glycol 400 (PEG), respectively).

Time Courses For Recovery From Dilation By Calcium Antagonists

To investigate the recovery time course from dilation effects of calcium antagonists, the following procedure was used for application and washout of all drugs. After a 5-minute exposure to the minimum dose of a drug that induced maximum dilution, the bath solution was changed three times, then once every 5 minutes thereafter. The course of change in vessel diameter over time was compared to that for adenosine, a well-known cerebral vasodilator.8,26 The doses used for this study were as follows: diltiazem 10⁻⁴ M, verapamil 10⁻⁵ M, nifedipine 10⁻⁶ M, nimodipine 10⁻⁶ M, and adenosine 10⁻³ M.

Drug Preparation

Care was taken to avoid light-inactivation of nifedipine and nimodipine. Diltiazem, verapamil, and adenosine were dissolved in PSS. Ethanol was the solvent for nifedipine (10⁻³ M) and PEG for nimodipine (10⁻³ M). Further dilutions (10⁻⁵ to 10⁻¹² M) were made with PSS.

Statistical Analysis

Group mean diameters for each drug dose, expressed as a percentage of control vessel diameter, were first compared by one-way analysis of variance (ANOVA) and subsequently by Student-Newman-Keuls multiple range test. 30 The level of significance was 0.05. Median effective dose (ED₅₀) values were calculated from probit transformations as described by Tallarida and Murray.26 Negative log ED₅₀ values were used to calculate the relative potencies of the different antagonists. Values are given as means ± standard error of the means.

Results

The passive arteriolar diameter, as determined for each vessel at the beginning of the experimental protocol in an extraluminal bath solution of pH 7.3 at 60 mm Hg transmural pressure and 24°C, averaged 78.2 ± 4.3 μm for 28 specimens. The control vessel diameter after development of spontaneous tone at 37°C averaged 52.3 ± 3.0 μm. When the extraluminal pH was changed, vessels dilated to 138% ± 2.3% at pH 6.8 and constricted to 73.6% ± 1.1% at pH 7.6. Vessels that showed weak responses (< 20% dilation at pH 6.8 or < 15% contraction at pH 7.6) were discarded at this stage. Control diameters and pH responses were not significantly different among vessels used to determine dose-response curves (Table 1) or recovery time courses (Table 2).

TABLE 1
Control diameters and pH responses of vessels used to determine dose-response curves

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control Diameter (μm)</th>
<th>Response (%) at pH:</th>
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<tbody>
<tr>
<td></td>
<td>6.8</td>
<td>7.6</td>
</tr>
<tr>
<td>diltiazem</td>
<td>43.7 ± 3.25</td>
<td>131.5 ± 3.38</td>
</tr>
<tr>
<td>verapamil</td>
<td>50.3 ± 7.11</td>
<td>142.4 ± 6.93</td>
</tr>
<tr>
<td>nifedipine</td>
<td>56.4 ± 8.56</td>
<td>142.5 ± 1.42</td>
</tr>
<tr>
<td>nimodipine</td>
<td>44.9 ± 6.28</td>
<td>138.7 ± 4.82</td>
</tr>
</tbody>
</table>

* Values are means ± standard error of the means. Response is expressed as percentage of control diameter.
† ANOVA = analysis of variance: p values in parentheses; ns = difference not significant.
Calcium antagonists dilated penetrating arterioles immediately after their application, inducing maximum effects within 2 to 3 minutes at each dose. These dilation responses were dose-dependent and significant for every calcium antagonist (ANOVA: p < 0.001). Maximum dilation for each calcium antagonist was as follows: diltiazem 46.4% ± 5.6%, verapamil 53.1% ± 6.0%, nifedipine 46.9% ± 6.1%, and nimodipine 47.1% ± 5.4% (Fig. 1). The magnitude of maximum dilation was not significantly different among these calcium antagonists (ANOVA: p = 0.845). Solvent vehicles had essentially no effect on the diameter of penetrating arterioles at concentrations corresponding to those used with nifedipine or nimodipine between 10⁻¹² and 10⁻⁶ M (Fig. 1C and D). Ethanol, however, significantly constricted the arterioles at the highest concentration, corresponding to 10⁻⁵ M nifedipine (ANOVA: p < 0.001). Nifedipine at 10⁻³ M constricted the vessels significantly when compared with 10⁻⁶ M (p < 0.05), and this constriction was comparable to that obtained with the solvent alone (Fig. 1C).

Sensitivity of penetrating arterioles to each calcium antagonist occurred in the following order: diltiazem 1.52 × 10⁻⁶ M (-log \( ED_{50} \): 6.12 ± 0.21 for six specimens), verapamil 1.08 × 10⁻⁷ M (-log \( ED_{50} \): 7.01 ± 0.09 for five specimens), nifedipine 8.65 × 10⁻⁹ M (-log \( ED_{50} \): 8.10 ± 0.09 for five specimens), and nimodipine 1.62 × 10⁻⁹ M (-log \( ED_{50} \): 8.98 ± 0.21 for seven specimens). These values were all significantly different from each other (p < 0.01).

**Time Courses For Recovery From Dilation By Calcium Antagonists**

Recovery time for dilation effects of calcium antagonists after washout, as compared with that of adeno-
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e, is shown in Fig. 2. Time for return to the resting
diameter (100% ± 5%) with each drug was as follows:
adenosine 8.5 ± 1.0 minutes, diltiazem 15.5 ± 1.8
minutes, verapamil 12.5 ± 1.8 minutes, nifedipine 19.0
± 3.9 minutes, and nimodipine 30.0 ± 1.6 minutes.
Recovery time was significantly longer with diltiazem
(p < 0.05) and with nifedipine and nimodipine (p >
0.01) than with adenosine. Recovery time was also
significantly longer with nimodipine than with the other
calcium antagonists (p < 0.01).

Discussion

The present study demonstrates that four calcium
antagonists (diltiazem, verapamil, nifedipine, and ni-
modipine) significantly dilate intracerebral penetrating
arterioles. Dilation persists for a significantly longer
time after washout with diltiazem, nifedipine, and ni-
modipine than with adenosine, while recovery time
with verapamil was not significantly different from that
with adenosine. This is the first direct examination of
the effects of calcium antagonists on intracerebral pen-
etrating arterioles, the most distal resistance vessels in
the cerebral microcirculation.

Few in vitro studies of the effects of calcium antag-
onists on resting (nonactivated) diameter or tone have
been done, except for pial arteriole studies using in vivo
cranial window methods. In larger arteries, the effects
of calcium antagonists have been studied by their an-
ticontractile effects to various vasoconstrictors or their
vasodilatory effects after precontraction by agonists.2,24
The magnitude of vasodilation in cranial window studies
in vivo is reported most frequently to be less than
25%; 6% caused by 10^-4 M verapamil in rats,4 15% by
10^-5 M verapamil and 6% by 10^-7 M nimodipine in
mice,23 and 21% by 10^-5 M nimodipine in cats.5 One
exceptional study in cats showed a 33% dilation caused
by 6 x 10^-5 M nifedipine and a further dilation of
about 80% by 10^-3 M nifedipine.11 This study is the
only one that used the microinjection technique for the
application of drugs. In our study of penetrating arte-
rioles in rats, the magnitude of vasodilation was about
50% for each calcium antagonist.

The duration of vasodilation of pial arterioles by
calcium antagonists was also investigated in cats by
Auer and Mokry.6 They found that vessel diameters
returned to 5% above resting levels within 15 minutes
after superfusion of 2.4 x 10^-5 M nimodipine for 10
minutes. The vessels in our study took 30 minutes to
return to 5% above resting diameter after washout of
as little as 10^-6 M nimodipine applied for 5 minutes.
Recovery time seems to depend on the concentration
and duration of application of calcium antagonists. In
the present study, after cumulative long-term applica-
tions of calcium antagonists, recovery times approaching
1 hour were necessary for the arterioles to return to
resting diameter. Recovery time also depends on the
calcium antagonists involved: nimodipine had the most
prolonged effect of the four calcium antagonists stud-
ied, and diltiazem and nifedipine had effects of longer
duration than adenosine. These differences in recovery
time may be due to a different affinity to receptor sites,
and could also be related to lipid solubility, because
lipid-soluble drugs such as nifedipine and nimodipine
had longer recovery times than water-soluble drugs such
as diltiazem and verapamil.

There are significant interspecies differences in the
responsiveness of cerebral arteries to calcium antago-

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<th>Drug</th>
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<th>Response (%) at pH:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>6.8</td>
</tr>
<tr>
<td>adenosine</td>
<td>56.0 ± 3.59</td>
<td>137.4 ± 3.87</td>
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<tr>
<td>diltiazem</td>
<td>49.7 ± 6.74</td>
<td>145.6 ± 7.87</td>
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<tr>
<td>verapamil</td>
<td>61.6 ± 4.92</td>
<td>135.2 ± 4.16</td>
</tr>
<tr>
<td>nifedipine</td>
<td>60.9 ± 4.68</td>
<td>137.2 ± 4.22</td>
</tr>
<tr>
<td>nimodipine</td>
<td>49.8 ± 6.83</td>
<td>145.6 ± 7.89</td>
</tr>
<tr>
<td>ANOVA†</td>
<td>ns (0.384)</td>
<td>ns (0.585)</td>
</tr>
</tbody>
</table>

* Values are means ± standard error of the means. Response is
expressed as percentage of control diameter.
† ANOVA = analysis of variance; p values in parentheses; ns =
difference not significant.

nists. For example, Nosko, et al., have shown that nimodipine causes varying degrees of vasodilation when applied to the basilar artery of dog, monkey, and man. Caution should also be used in extrapolating from in vitro vascular preparation data to mechanisms of cerebral blood flow control in the intact human. Nonetheless, the present data suggest that, at least in rats, calcium antagonists cause dilation of intracerebral arterioles of greater amplitude and duration than that seen in pial arterioles. Several characteristic differences are noted in intracerebral penetrating arterioles as compared with the other vessels in the cerebral circulation. First, the penetrating arterioles have smaller vessel diameters, usually less than 100 μm. Second, morphologically, the wall consists of only one layer of smooth muscle cells and a thin adventitial layer of leptomeningeal sheath, with a layer of endothelial cells. Third, neuropharmacological differences are noted. Sympathetic innervation of the smaller intraparenchymal vessels is thought to be associated with a catecholamine system arising in the locus ceruleus, while the larger cerebral vessels are innervated by nerves arising in the superior cervical ganglion. Heterogeneity in the distribution of perivascular neuropeptides in cerebral vessels has recently been reported. Immunoreactivity to neuropeptide Y was particularly abundant around the major arteries of the circle of Willis, and could be readily demonstrated in pial arterioles on the convexity of the cerebral cortex, but was noted only occasionally around penetrating arterioles.

The reason for the more pronounced effect of calcium antagonists on the penetrating arterioles is not clear. One possible explanation might be that penetrating arterioles have smaller diameters, given the fact that dilation responses to calcium antagonists become significantly greater with decreasing arteriolar size in pial arterioles. Adenosine produced an almost twofold increase in the dilation responses to calcium antagonists in intracerebral arterioles as compared with the other vessels in the cerebral circulation. First, the penetrating arterioles have smaller vessel diameters, usually less than 100 μm. Second, morphologically, the wall consists of only one layer of smooth muscle cells and a thin adventitial layer of leptomeningeal sheath, with a layer of endothelial cells. Third, neuropharmacological differences are noted. Sympathetic innervation of the smaller intraparenchymal vessels is thought to be associated with a catecholamine system arising in the locus ceruleus, while the larger cerebral vessels are innervated by nerves arising in the superior cervical ganglion. Heterogeneity in the distribution of perivascular neuropeptides in cerebral vessels has recently been reported. Immunoreactivity to neuropeptide Y was particularly abundant around the major arteries of the circle of Willis, and could be readily demonstrated in pial arterioles on the convexity of the cerebral cortex, but was noted only occasionally around penetrating arterioles.

The reason for the more pronounced effect of calcium antagonists on the penetrating arterioles is not clear. One possible explanation might be that penetrating arterioles have smaller diameters, given the fact that dilation responses to calcium antagonists become significantly greater with decreasing arteriolar size in pial arterioles. Adenosine produced an almost twofold increase in the diameter of rat pial arterioles, which appears to be greater than the dilation seen with penetrating arterioles in our study (about 40% increase in diameter by 10^{-3} M adenosine). It does not seem to be greater than the dilation seen with penetrating arterioles in our study (about 40% increase in diameter by 10^{-3} M adenosine). It is likely that vasodilation of penetrating arterioles by calcium antagonists would simply be the result of their higher basal tone compared to that of pial arterioles. Alternatively, the tone of penetrating arterioles might be more dependent on extracellular calcium.

In our experiments with penetrating arterioles, a 60% dilation was produced by calcium-free media containing 0.5 mM EDTA. The vessels rapidly returned to their control diameter, usually within 3 minutes after the bath solution was changed to calcium-containing media (unpublished data from our laboratory). Thus, the long-lasting effects of calcium antagonists on penetrating arterioles may be due to their higher affinity to receptor sites of penetrating arterioles than those of pial arterioles, and may not be due to failure to reestablish intracellular calcium pools once they are depleted.

Our data suggest that the spontaneous tone of intracerebral arterioles is highly dependent on extracellular calcium. This appears to be different from the myogenic tone of larger cerebral arteries, which was independent of extracellular calcium in studies conducted with diltiazem by Bevan. The ED_{50}'s of intracerebral penetrating arterioles in response to the four calcium antagonists were 1.52 x 10^{-6} M (diltiazem), 1.08 x 10^{-7} M (verapamil), 8.65 x 10^{-9} M (nifedipine), and 1.62 x 10^{-9} M (nimodipine). These values are quite similar to those found in larger cerebral arteries. Ethanol, a solvent for nifedipine, significantly constricted penetrating arterioles at a concentration corresponding to 10^{-5} M nifedipine (22%). It is likely that the vasoconstriction observed at 10^{-5} M nifedipine is due to the effect of the solvent. Our result with ethanol is comparable to that found by Altura, et al., in larger cerebral arteries and pial arterioles, although a higher dose was required to constrict those vessels; in our study 10^{-4} M nifedipine could not eliminate the constriction by ethanol.

Various calcium antagonists have recently become available for the treatment of cerebral arterial vasospasm, which is an important cause of morbidity and mortality in patients with SAH. Clinical studies increasingly suggest that calcium antagonists do exert a beneficial effect on the overall course of the sequelae of SAH. Despite these encouraging results, recent clinical and laboratory evidence suggests that systemic therapy with calcium antagonists may not significantly affect the degree of angiographically visualized vasospasm. For example, Ljunghagen, et al., concluded that, in patients undergoing early surgery after SAH, neurological deficits due to spasm were lessened by nimodipine therapy, whereas the degree of angiographically defined vasospasm was unchanged. Our data may help to explain this apparent discrepancy between angiography and clinical findings in patients with cerebral vasospasm who are undergoing treatment with calcium antagonists. It appears that intracerebral arterioles are more sensitive to calcium antagonists than are angiographically visible larger cerebral arteries, and may be responsible for decreased total cerebrovascular resistance despite persistent contraction of more proximal vessels within the subarachnoid space. More data are needed before this information on relatively normal intracerebral arterioles can be extrapolated to arterioles affected by SAH.

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