The Selective Inhibition of Serotonin-Induced Contractions of Rabbit Cerebral Vascular Smooth Muscle by Calcium-Antagonistic Dihydropyridines

An Investigation of the Mechanism of Action of Nimodipine

ROBERTSON TOWART

SUMMARY I studied the role of calcium in the activation of isolated rings of saphenous and basilar arteries of the rabbit by comparing the effect of calcium withdrawal with the effect of the calcium antagonist nimodipine [isopropyl(2-methoxyethyl)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate]. Serotonin-induced contractions of both vessels were inhibited quickly by incubation in calcium-free solution, showing the contractions of both vessels to be dependent on extracellular calcium. Potassium-induced contractions of both vessels were inhibited markedly by nimodipine (basilar: ID₅₀ = 1.7 × 10⁻¹⁰ mol/liter; saphenous: ID₅₀ = 2.6 × 10⁻¹⁰ mol/liter), showing depolarization-induced calcium influx (probably through "potential sensitive channels", PSCs) to be sensitive to nimodipine. In the basilar artery the sustained, tonic phase of serotonin-induced contractions (probably through "receptor operated channels," ROCs) was potently inhibited (ID₅₀ = 7.3 × 10⁻¹⁰ mol/liter) by nimodipine. However, the initial short-lived phase of this contraction of the basilar artery was relatively unaffected (ID₅₀ = 2 × 10⁻⁶ mol/liter), indicating that the inhibitory action of nimodipine on tonic contractions of the basilar artery was not due to antagonism at the serotonin receptor level. In contrast, in the saphenous artery the serotonin-induced contractions were unaffected by nimodipine in doses up to 2.4 × 10⁻⁵ mol/liter. It is postulated that the selective inhibition of the sustained tonic contraction of the basilar artery is due to a selective inhibition by nimodipine of calcium movement through ROCs in this vessel. Agonist-induced activation of ROCs in peripheral blood vessels does not seem to be affected by calcium antagonists. Circ Res 48: 650-657, 1981

IN the past decade a diverse group of drugs, the "calcium antagonists," (Fleckenstein, 1977) has found a place in therapy of many cardiovascular disorders. The mechanism of action of these substances, typified by verapamil and nifedipine (Vater et al., 1972), has been postulated to be blockade of transmembrane calcium influx (Fleckenstein et al., 1967). This mechanism has been shown for cardiac muscle (Kohlhardt et al., 1972a, 1972b; Ehara and Kaufmann, 1978; Kohlhardt and Fleckenstein, 1977), vascular smooth muscle (Karaki and Weiss, 1979; Thorens and Haeusler, 1979), and several other tissues (see Rosenberger and Triggle, 1978).

Recent reports have shown that cerebral vascular smooth muscle is more sensitive to the effects of some vasodilators of the calcium antagonist group than is peripheral vascular smooth muscle (Hayashi and Toda, 1977; Allen and Banghart, 1979; Shimizu et al., 1980). Thus cerebral vasodilation with these compounds may be possible without a fall in systemic blood pressure. This selective action is not shown by papaverine, nitrates, or adenosine (Toda, 1974; Hayashi and Toda, 1977).

Calcium antagonists have been found to be potent inhibitors of vasospasm, both in the coronary (Hillis and Braunwald, 1978; Théroux et al., 1979) and, more recently, in the cerebral circulation (Allen and Bahr, 1979; Edvinsson et al., 1979; Takagi et al., 1979). Calcium antagonists with powerful actions on cerebral vessels but with little effect on arterial pressure may therefore provide rational therapy for cerebrovascular disorders that involve reduced perfusion or vasospasm. With this in mind, the substance nimodipine (Kazda and Hoffmeister, 1979; Towart and Kazda, 1979), a derivative of the calcium antagonist nifedipine, has been developed. Nimodipine (Fig. 1) has cerebral vasodilator actions at doses that do not decrease systemic blood pressure (Kazda et al., 1979) and has protective actions in several animal models of cerebral ischemia (Hoffmeister et al., 1979; Kazda and Hoffmeister, 1979) or hypoxia (Hoffmeister et al., 1979).

Stimulation of smooth muscle by depolarization and by receptor activation use different sources of activator calcium (see Bolton, 1979). Selective effects of calcium antagonists have been ascribed to
SELECTIVE INHIBITION OF CEREBRAL VESSELS

Towart

The structure of nimodipine.

different effects of the agents on calcium sources (Schumann et al., 1975; Allen and Banghart, 1979). To investigate the apparently selective effects of calcium antagonists on cerebral vascular smooth muscle, we have compared the effects of nimodipine on potassium- and serotonin-induced contractions of isolated basilar and saphenous arteries.

Methods

Male or female Chinchilla rabbits (3-4 kg) were killed by pentobarbital overdose (60 mg/kg, iv). A length of one saphenous artery was removed, and the brain was exposed by removing the cranium. The basilar artery was carefully removed from the underside of the brain, and both arteries were dissected free of connective tissue and fitted, using the method of Edvinsson et al. (1974), to two wire holders. The vessels immediately were suspended in 20-ml organ baths containing Krebs-Henseleit solution (composition: NaCl, 119 mm; KCl, 4.8 mm; CaCl₂, 2.5 mm; KH₂PO₄, 1.2 mm; MgSO₄·7H₂O, 1.4 mm; NaHCO₃, 25 mm; glucose, 11 mm; Na₂EDTA, 13.4 µM) at 37°C gassed with 95% O₂-5% CO₂. The pH of the solution was 7.2-7.4. The tension of the vessels was measured with Statham UC 2 strain gauges connected to a DAS 10/4 data acquisition system (Fa. IFD, Mühlheim a.d. Ruhr, FRG). The resting tension was adjusted to 500 mg. An interval of at least an hour was allowed for equilibration, after which concentration response curves to potassium chloride and to serotonin were obtained.

Depolarization-Induced Contractions

Submaximal contractions were induced by adding KCl to the bath to give a final concentration of 45.6 mmol/liter. After the contractions had stabilized, nimodipine or the corresponding concentration of DMSO (the solvent used for nimodipine) was added cumulatively, allowing time between additions for stabilization of relaxation; DMSO in the concentrations used had no effect on the contractions.

Serotonin-Induced Contractions

Submaximal contractions were induced by addition of serotonin (final concentration = 2.5 × 10⁻⁷ mol/liter) to the bath for 4 minutes. An interval of 20 minutes was allowed for washing. At least four reproducible contractions (±15%) were induced in the presence of DMSO before addition of nimodipine.

Contractions Due to Serotonin-Induced Calcium Influx

Vessels were incubated in nominally calcium-free solution (see below) for 12 minutes. The addition of serotonin (2.5 × 10⁻⁷ mol/liter) after 8 minutes of the incubation period produced only transient contractions, but subsequent replacement of calcium (2.5 mmol/liter as CaCl₂) at end of the incubation period produced sustained contractions. The procedure of calcium withdrawal and replacement after 12 minutes in the absence of serotonin produced only slight and transient increases in tone in the basilar artery, and none in the saphenous artery. Thus the sustained contractions obtained by calcium replacement in the presence of serotonin may be regarded as being due to serotonin-stimulated calcium influx.

EDTA (5 µg/ml) was added routinely to the Krebs-Henseleit solution as an anti-oxidant.

FIGURE 1

Potassium depolarization (addition of KCl ∆; total bath K⁺ contraction = 45.6 mmol/liter) contracted both the saphenous (SAPH) and basilar (BAS) artery rings. The cumulative addition of nimodipine (○, 2.4 × 10⁻¹⁰ M – 2.4 × 10⁻⁶ M) relaxed both vessels completely.
In "calcium withdrawal" experiments, CaCl₂ was replaced with NaCl, giving a buffered Ca²⁺ concentration of about 10⁻⁵ mol/liter. Complete removal of Ca²⁺ by increasing the EDTA concentration was found to have a deleterious effect on the vessels.

Because of the light sensitivity of nimodipine, experiments with the compound were carried out under sodium light, and control contractions were induced in the presence of the appropriate concentration of DMSO which was used to dissolve the drug.

Results are expressed as means ± SE. Comparisons of the results were made using Student's t-test. Fifty percent inhibitory doses (ID₅₀) have been estimated by calculating, by the method of least squares, the line of best fit and 95% confidence limits of the descending portion of the log dose-response curve (Diem and Lentner, 1970). The ID₅₀ and its confidence limits were then obtained graphically (see Fig. 5). Comparisons of regression lines and their slopes also were carried out according to Diem and Lentner (1970).

**Results**

**K⁺-Depolarization**

Increase of the K⁺ concentration to 45.6 mmol/liter produced immediate contractions of both vessels (see Fig. 2; Table 1). Cumulative addition of nimodipine thereafter relaxed both vessels dose-dependently, with maximum relaxation (basilar artery 109%, saphenous artery 102% of the potassium-induced contraction) occurring with nimodipine 2.4 x 10⁻⁸ mol/liter. Although the basilar artery relaxed more quickly than the saphenous artery (Fig. 2), the 50% inhibitory doses (ID₅₀) of nimodipine for the two vessels and the slopes of the concentration response curves (Table 1) were not statistically different.

**Serotonin-Induced Contractions**

Addition of serotonin (2.5 x 10⁻⁷ mol/liter) to the organ bath produced immediate contraction of both vessels. In the basilar artery, two components of contraction could be distinguished, i.e., a steep rise in tension, sometimes followed by a transient relaxation, with a subsequent slow rise in tension to a sustained plateau.

The maximum serotonin-induced contractions
Table 1 Effect of Nimodipine on Isolated Rabbit Basilar and Saphenous Arteries Contracted with K+ (45.6 mmol/liter)

<table>
<thead>
<tr>
<th>Artery</th>
<th>n</th>
<th>Contraction induced by K+ (g)</th>
<th>Maximum relaxation induced by nimodipine (2.4 × 10⁻⁷ mol/liter)</th>
<th>Regression line of cumulative dose-response curve to nimodipine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basilar</td>
<td>4</td>
<td>1.60 ± 0.21 g</td>
<td>1.75 ± 0.26 g (100%)</td>
<td>ID₅₀ 1.7 × 10⁻⁵ mol/liter 0.82-2.8 × 10⁻¹⁰ r 0.92 42%†</td>
</tr>
<tr>
<td>Saphenous</td>
<td>4</td>
<td>3.54 ± 0.28 g</td>
<td>3.61 ± 0.24 g (102%)</td>
<td>ID₅₀ 2.6 × 10⁻⁷ mol/liter 1.6-4.3 × 10⁻⁹ r 0.95 41%‡</td>
</tr>
</tbody>
</table>

n = the number of preparations used.
† These values are not significantly different.
‡ The regression lines are parallel (P < 0.05).

were greater in the saphenous artery, but relative to the contractions produced by K⁺, 55.6 mmol/liter, the basilar artery was more responsive at low concentrations of serotonin (Fig. 3). A concentration of 2.5 × 10⁻⁷ mol/liter for serotonin was chosen as a standard, submaximal concentration.

Nimodipine in concentrations up to 2.4 × 10⁻⁵ mol/liter had no effect on the serotonin-induced contractions of the saphenous artery (Figs. 4 and 5). The initial phasic component of the serotonin-induced contraction of the basilar artery also was little affected by nimodipine (Fig. 4), being inhibited only at the highest concentration tested (Fig. 5). The sustained tonic component of the serotonin-induced contraction of the basilar artery was, however, potently inhibited by nimodipine (Fig. 4). The individual results from such experiments produced a concentration-response curve (Fig. 6) from which the ID₅₀ of nimodipine was calculated to be 7.3 × 10⁻¹⁰ mol/liter.

Calcium Withdrawal

Because complete withdrawal of extracellular calcium by EGTA was found in preliminary experiments to be deleterious to the vessels, this technique was not used. The nominally "calcium-free" solution used (see Methods) had a free calcium

**Figure 5** Summarized results of 19 of the experiments shown in Figure 4. Nimodipine potently inhibits the sustained tonic component (●) of the serotonin-induced contraction of the basilar artery, whereas the initial phasic component (○) is affected only at much higher concentrations. The serotonin-induced contractions of the saphenous artery (▲) are unaffected by nimodipine.

**Figure 6** Percentage inhibition of the slow component of the serotonin-induced contraction of the isolated rabbit basilar artery produced by nimodipine. From the line of best fit (r = 0.67; n = 13; b = -20) the ID₅₀ was graphically calculated to be 7.3 × 10⁻¹⁰ mol/liter (95% confidence limits 1.4 × 10⁻¹⁰-2.4 × 10⁻⁹ mol/liter).
concentration of around $10^{-5}$ mol/liter, and its effects on serotonin-induced contractions are shown in Figure 7. The contractions of both vessels were markedly inhibited by the treatment. The cumulative results (Fig. 8) show that the sustained tonic component of contraction of the basilar artery was lost immediately. The apparently exponential fall of the contractions of the saphenous artery and of the fast phasic component of the basilar artery with time indicate the basic dependence of these contractions on physiological concentrations of extracellular calcium, but suggest the presence of limited intracellular calcium stores in the vessels. These are, however, not large enough to explain the lack of effect of nimodipine on the saphenous artery (Fig. 4), or to explain the qualitative difference in sensitivity to nimodipine of the two vessels.

**Serotonin-Stimulated Calcium Influx**

Preliminary experiments were carried out to separate effects due to serotonin-induced release of intracellular calcium from serotonin-induced stimulation of calcium influx.

Under the conditions of frequent washing, neither the basilar nor the saphenous artery develop active tension in Krebs-Henseleit solution containing 2.5 mM Ca$^{2+}$. However, after exposure to serotonin under nominally calcium-free conditions, the replacement of 2.5 mmol/liter calcium resulted in sustained and reproducible contractions (Figs. 9 and 10). These can therefore be regarded as contractions due to serotonin-stimulated calcium influx.

Nimodipine in the highest concentration tested ($2.4 \times 10^{-7}$ mol/liter) had no effect on the contractions produced by serotonin-stimulated calcium influx in the saphenous artery (Fig. 9) but, in much lower concentrations, inhibited the contractions of the basilar artery produced in this way (Fig. 10). Preliminary concentration response curves indicate an ID$_{50}$ of around $2 \times 10^{-9}$ mol/liter.

**Discussion**

Nimodipine, a new calcium antagonist, is a potent inhibitor of depolarization-induced contractions of peripheral and cerebral vascular smooth muscle. Its effects on serotonin-induced contractions of the vessels are different, however. The contractions of the saphenous artery were unaffected by nimodipine, whereas the sustained phase of contraction of the basilar artery was markedly inhibited (Figs. 4, 5). These results extend reports that other calcium antagonists selectively inhibit agonist-induced contractions of canine cerebral vessels (Hayashi and Toda, 1977; Allen and Banghart, 1979; Shimizu et al., 1980).

It appears that contractions of vascular smooth muscle induced by calcium or potassium depolarization are potently inhibited by calcium antagonists, whereas contractions induced by agonists, especially norepinephrine, are refractory to these drugs (Schumann et al., 1975; for review see Bolton, 1979). This observation has often been explained by the hypothesis that norepinephrine acts by releasing an “intracellular pool” of calcium (Hudgkins and Weiss, 1968). Contractions induced by norepinephrine, according to this theory, are not inhibited by calcium antagonists, because no transmembrane calcium influx would be required. Recent work has shown, however, that agonists such as norpinephrine...
Agonist-induced influx of calcium is different in many ways from depolarization-induced calcium influx. The existence of two different populations of calcium channels, i.e., receptor operated channels (ROCs) and potential sensitive channels (PSCs), therefore has been postulated (Bolton, 1979). The PSCs seem to be sensitive to organic calcium antagonists, but in several types of vascular smooth muscle ROCs are insensitive to these drugs. The potent effects of these agents on K⁺-depolarization-induced contractions of vascular smooth muscle therefore is due to block of PSCs. The lack of effect on agonist-induced contractions in vascular smooth muscle then is due to (1) agonist-induced release of intracellular calcium to initiate the contraction, followed by (2) influx of calcium via the unaffected ROCs. Influx of calcium maintains contraction and allows repletion of intracellular calcium stores despite continued presence of the calcium antagonist.

This hypothesis explains effects of nimodipine that have been obtained in peripheral vessels. Serotonin-induced contractions in calcium-free solutions are reduced quickly (Figs. 7 and 8) due to lack of calcium influx and to reduced refilling of intracellular stores for subsequent contractions. K⁺-induced contractions of aorta (Towart and Kazda, 1979) and of the saphenous artery (Fig. 2) are readily inhibited by nimodipine, due to block of the PSCs. Norepinephrine-induced contractions of aorta (Towart and Kazda, 1979) and serotonin-induced contractions of the saphenous artery (Figs. 4, 5) are unaffected because nimodipine has no effect on ROCs in these vessels. This was also demonstrated by the preliminary experiments shown in Figure 9. Under calcium-free conditions, serotonin induced a small contraction (intracellular calcium) which rapidly decayed. Subsequent addition of calcium allows the ion to enter through the already open ROCs, producing a contraction. Such contractions were not affected by nimodipine (Fig. 9).

In the basilar artery, the PSCs are also highly sensitive to nimodipine, as demonstrated by complete block of K⁺-induced contractions. The lack of effect on intracellular calcium is shown by failure of nimodipine to alter the fast phase of contraction except at the highest doses. The sustained tonic phase of contraction is, however, potently inhibited by the drug.

There are at least three possible explanations (which follow) for the marked differences between the two vessels:

1. Different sources of activator calcium. Allen and Banghart (1979) have explained their findings with nifedipine by postulating that the basilar artery uses primarily extracellular calcium, and the femoral artery uses primarily intracellularly bound calcium to provide agonist-induced contractions. Our experiments with calcium-free solution showed that both vessels are highly dependent on extracellular calcium. However, the first phase of the con-
traction of the basilar artery was, if anything, more resistant (Fig. 8) to calcium withdrawal than the contractions of the saphenous artery. This, and the resistance of phasic contraction to calcium antagonist (Fig. 4) observed in the basilar artery, suggest a significant intracellularly bound pool of calcium in the basilar artery. Also in the "calcium replacement" experiments (Figs. 9 and 10), both vessels behaved similarly. The short-lived spike after addition of serotonin, with a sustained contraction only after addition of calcium, suggests that the basic mechanism in both vessels is similar, i.e., quick release of intracellular calcium with sustained contraction due to agonist-induced activation of ROCs.

2. Differences in activation of PSCs. There is much evidence for pharmacomechanical coupling in vascular smooth muscle [i.e., contractions are produced by agonists without marked depolarization (Somlyo and Somlyo, 1968, 1970)]. There also exists the possibility that certain agonists may act on the basilar artery by depolarization or by causing an increase in spike activity. Serotonin in this vessel could therefore operate PSCs, not ROCs, which could explain the sensitivity to nimodipine. Although this possibility cannot be excluded without electrophysiological studies, preliminary experiments have shown that KCl- and serotonin-induced contractions of the basilar artery under these conditions are additive, which provides evidence that serotonin does not activate the vessel solely by depolarization.

3. Differences in sensitivity of ROCs to calcium antagonists. The most likely explanation in my view is that the serotonin activation in the basilar artery also involves ROCs, but that these are, in contrast to those of peripheral vessels, sensitive to calcium antagonists. The experiments shown in Figure 10 demonstrate contractions produced by serotonin-induced influx of calcium. The contractions clearly are inhibited by nimodipine, and the effective concentration range seems to be similar to that of the detailed study under more usual conditions.

Some findings also suggest that sensitivity of ROCs to calcium antagonists varies from vessel to vessel. The calcium antagonist cinnarizine has been reported to antagonize norepinephrine-induced contractions of the rabbit mesenteric artery but not those of the rabbit aorta (Broekaert and Godfraind, 1979). Cinnarizine was found to block norepinephrine-induced influx of extracellular calcium into the mesenteric artery but not into the aorta, leading to the suggestion that the norepinephrine-activated channels in the two vessels may be different. In large coronary arteries norepinephrine also was found to induce contraction by stimulating calcium influx, and such contractions were found to be more sensitive to the calcium antagonists D 600 or SKF 525a (proadifen) than similar contractions induced in peripheral vessels (van Breemen et al., 1978). In other smooth muscles the agonist-induced contractions of the guinea-pig ileum and of the rat vas deferens show distinct variations in their sensitivity to calcium antagonists, although both tissues also are sensitive to removal of extracellular calcium (Triggle et al., 1979).
My hypothesis therefore is that there are distinct differences in tissue sensitivity to calcium antagonists, and that the observed sensitivity of cerebral vessels to calcium antagonists such as nimodipine is due to a selective block of the ROCs in these vessels.

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R Towart

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