Dissociation Between Calcium Influx Blockage and Smooth Muscle Relaxation by Nifedipine in Spontaneously Hypertensive Rats

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Aortic smooth muscle isolated from spontaneously hypertensive rats (SHR) and normotensive, age-matched Wistar Kyoto rats (WKY) was precontracted by potassium chloride. The relaxant effect of nifedipine (NIF) was much more pronounced in SHR than in WKY, while the relaxation produced by nitroglycerin (NTG) was similar in both tissues. EC_{50} values were (in - log M) NIF: SHR 13.1 ± 0.4 and WKY 9.4 ± 0.2 (p<0.05); NTG: SHR 7.35 ± 0.3 and WKY 7.26 ± 0.18 (NS). Aortas from SHR were less sensitive to the contractile effect of Ca^{2+} than their WKY controls (EC_{50} was 5.18 ± 0.03 in WKY and 2.76 ± 0.13 in SHR, p<0.05). The relaxant effect of NIF was dissociated from its effect on Ca^{2+} influx in SHR aortas. NIF 10^{-10} M relaxed the muscle by 100% without producing Ca^{2+} influx blockage, and NIF 10^{-9} and 10^{-8} M induced Ca^{2+} influx blockage while the muscle continued in the relaxed state. Chemically skinned aortic fibers from SHR were less sensitive to the contractile effect of Ca^{2+} than their normotensive (NR) controls (pCa for EC_{50} was 5.91 ± 0.05 in SHR and 6.20 ± 0.03 in NR, p<0.05). NIF 10^{-10} M depressed the contractile response to Ca^{2+} significantly more in SHR than in NR skinned fibers (pCa for EC_{50} for 5.62 ± 0.09 in SHR and 6.07 ± 0.07 in NR, p<0.05). These data suggest that 1) the more pronounced relaxant action of NIF on SHR aortas cannot be explained by its effect on Ca^{2+} influx, since both effects were dissociated in these vessels; and 2) the mechanism for NIF-dependent relaxation in SHR appears to be located not at the sarcolemmal but at some intracellular level. In this respect, a reduced calmodulin content in SHR arteries, along with an anticalmodulin action of NIF, could explain both the lesser sensitivity to Ca^{2+} and the greater sensitivity to NIF that was found in SHR aortas. (Circulation Research 1987;60:367-374)

Calcium influx blockers are used in the treatment of hypertension because of their vasodilating effects. These compounds decrease blood pressure in hypertensive patients while no significant effect in normotensive patients has been reported. An increased sensitivity of smooth muscle of hypertensive rats to some of these compounds has been reported. Nifedipine, diltiazem, and nisoldipine were reported to have an increased action on smooth muscle of hypertensive animals.

Recent experiments indicate that the supersensitivity of the smooth muscle of hypertensive animals to nifedipine is a function of the level of arterial pressure and is independent of the mechanisms that elevated arterial pressure.

The present study was undertaken to further characterize the relaxant effect of nifedipine, a widely used dihydropyridine, on the smooth muscle of normotensive and hypertensive rats. Results indicate that nifedipine exhibits a greater relaxant effect on vascular smooth muscle of hypertensive animals than on normotensive ones and that this action is not mediated through calcium influx blockage.

Materials and Methods

The experiments were performed in male, 4-month-old spontaneously hypertensive rats (SHR) of the Okamoto-Aoki strain. Age- and sex-matched normotensive Wistar-Kyoto rats (WKY) were used for controls. In the experiments performed on skinned fibers, normotensive Wistar rats from our own colony (NR) (and in a few experiments WKY) were used as controls. Systolic blood pressure was repeatedly measured in the tail by means of a pneumatic transducer (Narco Bio-Systems, Texas). Blood pressures just before death were 203 ± 10 mm Hg (SHR), 126 ± 2 mm Hg (NR), and 141 ± 6 mm Hg (WKY).

Rats were anesthetized with sodium pentobarbital, and a section of the aortas extending from the heart to the emergence of the renal arteries was removed. The vessels were placed in a bathing medium (PSS) of the following composition (mM): NaCl, 118; KCl, 5.32; NaHCO3, 1.34 and glucose, 5.6. The PSS was aerated with a gas mixture of 5% CO2 and 95% O2. The surrounding adipose tissue was gently removed, and two 2-mm-wide rings were cut from the abdominal end of the aorta and used for the mechanical experiments. The rest of the vessel was used for determination of calcium fluxes. The rings were transferred to organ baths filled with PSS at 37°C. They were rigidly suspended
from a Grass FT.03C force transducer whose output was fed into an oscillographic recorder. Resting tension was adjusted to 2 g by stretching the rings, and a stabilization period of 60 minutes was allowed before intervention.

The relaxant effect of nifedipine and nitroglycerin was studied in rings contracted by exposure to a solution with 35 mM KCl and an equimolar reduction of sodium chloride (K-PSS). The potassium-chloride concentration used was selected to produce a submaximal contracture of the vessel and minimal catecholamine release from nerve endings. The response to potassium chloride was not significantly different between the aortic smooth muscle from SHR and WKY. EC\textsubscript{50}s were 27.4 ± 1.4 mM in SHR and 28.0 ± 2.8 mM in WKY. The maximal tensions developed were 3.66 ± 0.42 g/mm\textsuperscript{2} in SHR and 3.91 ± 0.52 g/mm\textsuperscript{2} in WKY. After the contracture stabilized (usually about 15 minutes), either nifedipine or nitroglycerin was cumulatively added to the bath from stock dilutions to obtain the desired concentrations. The relaxant effect of each concentration was measured after the tension stabilized and was expressed as a percentage of the maximal contracture, which was taken as 100%.

Contractile effects of calcium were studied in rings stabilized in K-PSS with no calcium. Calcium was then added from stock dilutions to obtain the desired concentrations, and the effect of each calcium concentration was recorded after the tension stabilized. Maximal tension attained was considered 100%. From the curve created by data from each experiment, the concentrations that produced definite percents of the maximal effect (from 10% to 90% in increments of 10) were interpolated. These effective concentrations (i.e., EC\textsubscript{10}, EC\textsubscript{20}, EC\textsubscript{50}, etc.) were then averaged and plotted as a function of the amount of relaxation obtained, with the standard errors drawn on the x-axis.

To normalize the forces developed by the rings, these were divided by the cross-sectional area (CSA). Force per unit of CSA (tension or stress) is referred to here as tension. To calculate CSA, the following equation was used: area = mass/density x length. A factor of 2 was considered, since in the rings the CSA was doubled.

Nifedipine was dissolved in a mixture of 20% ethanol and 80% polyethylene glycol. The solvent itself did not have any effect on calcium fluxes.

To measure calcium influx, aortic strips were incubated for 60 minutes in PSS buffered with Tris-hydrochloride instead of NaHCO\textsubscript{3} and bubbled with 100% O\textsubscript{2} at 37°C (pH 7.40). After this period, the incubation was continued under three conditions: 1) In control experiments, the incubation was continued in PSS for 60 minutes; 2) To determine potassium-induced calcium influx, a second group was incubated for 60 minutes in K-PSS; 3) To determine the effect of nifedipine on potassium-induced calcium influx, a third group was incubated for 60 minutes in K-PSS, but in the last 20 minutes nifedipine was added to the solution. Under these conditions, transmembrane calcium movements in the first two groups reached steady-state conditions. After these incubation periods, the strips were pulse-labelled with \textsuperscript{45}Ca for 3 minutes, and then a washout was performed by one of two different methods: The tissues were either washed for 30 minutes in ice-cold, calcium-free PSS with 2 mM EGTA; or the strips were washed for 120 minutes in a bathing solution containing (mM) LaCl\textsubscript{3}, 80.8 glucose, 11 Tris, 6; and kept at pH 6.8 at 2\degree C. At the end of the washout period, the strips were placed overnight in 2 ml of 5 mM EDTA in distilled water at room temperature. This method has been reported to provide complete extraction of Ca\textsuperscript{2+} from the tissue. The scintillation fluid was then added, and the CPM in each tube was determined. Tissue Ca\textsuperscript{2+} content was calculated and expressed in \textmu M/kgww/min. Two different washout methods were used to counteract the possibility that the widely used EDTA method did not provide a complete washout of Ca\textsuperscript{2+} from the extracellular compartment. Because of the good results reported with the lanthanum method, a group of experiments with this technique was also performed. Since in preliminary experiments the washout curve of the lanthanum method in rat aorta became monoeponential between 60 and 70 minutes, the 120-minute value was selected.

To measure calcium efflux, the aortic strips were incubated for 120 minutes in K-PSS with \textsuperscript{45}Ca. The strips were then quickly rinsed in nonradioactive K-PSS and transferred to a series of test tubes containing K-PSS with 2 mM EGTA and no calcium, at intervals from 5 minutes to a maximum of 110 minutes. The tissue was then removed, blotted, and treated with EDTA as in the influx experiments. The parameter used to describe the rate of exchange of Ca\textsuperscript{2+} is the fractional loss, k (min\textsuperscript{-1}). In a group of experiments, the arterial strips were exposed to 10\textsuperscript{-10} M nifedipine between 60 and 85 minutes of the washout. The purpose of using no-calcium EGTA in the washout period was to prevent \textsuperscript{45}Ca\textsuperscript{-45}Ca exchange. The K-PSS was used to study the effect of nifedipine on Ca\textsuperscript{2+} influx and efflux under identical potassium-chloride concentrations.

Chemically skinned aortas from SHR and WKY were prepared following the procedure described by Ruegg and Paul. The NRE were used as controls of SHR in these experiments because it was difficult to obtain age-matched WKY at that time. In additional experiments in which WKY were available, the comparison with SHR yielded similar results (Figure 5). The preparation procedure was as follows: 1-mm aortic rings were incubated in a solution containing (mM) 10 imidazole, 5 EGTA, 50 KCl, 150 sucrose, pH 7.4, for 30 minutes at 0°C. Then, the skinnning was achieved by incubating the preparation 30 minutes further in the same solution with the addition of 1% Triton x-100, 0.5 mM dithioerythritol (DTE), and 2 µg/ml leupeptin. The preparations were then incubated for 5 minutes in 50% glycerol solution (pH 6.7) containing...
(mM) 20 imidazole, 4 EGTA, 10 MgCl₂, 7.5 ATP, 1 NaN₃, 0.5 DTE, and 2 μg/ml leupeptin. This solution was then stored at -15°C overnight.

Each skinned ring was divided in halves, and a strip approximately 1.5 mm long was cut from one of the halves and mounted horizontally between an adjustable stainless steel rod and a fixed tension transducer (AME 801 E, Aksjeselkapet Mikro-elektronikk, Hor-}

Mardal, Norway) and stretched approximately 30%. The preparations were bathed in a "relaxing" solution containing (mM) 21 K⁺, 36 Na⁺, 10 Mg (total), 4 EGTA, 7.5 ATP, 20 imidazole, 35 Cl⁻, 1 azide, 10 phosphocreatine, and 10 μ/ml creatine phosphokinase (pH 6.7). After a stabilization period of 20 minutes, contraction was induced by partly replacing EGTA with Ca²⁺-EGTA to stepwise increase free Ca²⁺. The concentration of free Ca²⁺ was calculated by means of the calculator program provided by Fabiato and Fabiato.

The absolute stability constants used in the program are those compiled by the same authors. The force induced by the different Ca²⁺ concentrations was recorded until a "plateau" was reached. One strip obtained from the other half of the ring was then placed in the same chamber, but during the stabilization period it was exposed to nifedipine 10⁻⁶ M. Then a concentration-force relation to calcium was performed in the presence of the same concentration of nifedipine. The experiments with and without exposure to nifedipine were performed in random order. The EC₅₀ (Ca²⁺ concentrations necessary to obtain a given percent of the maximal force) were calculated as described for the concentration-relaxation curves. Cross-sectional areas of the skinned strips were calculated according to the same equation used in the intact preparations. Since the skinned bundles have a considerable surface-to-volume ratio, the direct wet weight determination becomes uncertain because of rapid water evaporation. Thus, the dry weight of the strip was first determined in a Cahn electrobalance, model 25. The wet weight was then obtained by multiplying the dry weight by a correction factor. This factor was obtained from control experiments in which large pieces of the same artery (whose weight was less affected by water evaporation) were weighed when wet and again after drying. As shown in "Results," the EC₅₀ to the relaxing effect of NIF was 10⁻⁷ M in SHR and 10⁻⁹ M in either NR or WKY. Doses of nifedipine 10⁻⁸ M and higher were selected to test the effect on calcium fluxes because 10⁻⁹ M was the highest dose without effect on calcium influx in spite of 100% of relaxation. The same dose was selected for the studies on skinned fibers, since in intact fibers 10⁻¹⁰ M nifedipine relaxes approximately 50% of the normotensive smooth muscles and 100% of the hypertensive smooth muscles in spite of not blocking calcium influx.

The data were expressed as mean ± standard error (SEM) for each experimental group. Comparison between groups was accomplished by Student's t test or analysis of covariance. A difference of p<0.05 was considered significant.

Results

Figure 1A shows concentration-relaxation curves to nifedipine in potassium-chloride-contracted aortic rings from SHR (n = 6) and WKY (n = 10). Maximal active tension (MT) with K-PSS was similar in WKY and SHR (inset). The relaxant effect of nifedipine, however, was much more pronounced in SHR than in WKY at all concentrations of nifedipine used. * = p<0.05 with respect to SHR. Panel B: Concentration-relaxation curves to nitroglycerin in K-PSS-contracted aortic rings from SHR (n = 8) and WKY (n = 8). Maximal active tension (MT) with K-PSS was similar in both groups of arteries (inset). Note that with this vasodilator, whose mechanism of action is other than calcium influx blockage, a similar relaxation is elicited in WKY and SHR rings.
FIGURE 2. Contractile response to Ca$^{2+}$ in potassium-depolarized aortic rings from SHR ($n = 6$) and WKY ($n = 10$). Note that any given Ca$^{2+}$ concentration elicits a greater contracture in WKY than in SHR. Maximal tension attained with calcium (MT) was similar in SHR and WKY (inset). * = $p<0.05$ with respect to WKY.

- log $|M|$ SHR, 13.1 ± 0.4 and WKY, 9.4 ± 0.2 ($p<0.05$). Concentration-relaxation curves to nitroglycerin in potassium-chloride-contracted aortic rings from SHR ($n = 8$) and WKY ($n = 8$) are illustrated in Figure 1B. The relaxant effect of nitroglycerin was similar in both groups at any of the concentrations employed, as was the force development in response to K-PSS (inset). $EC_{50}$ were ($-\log |M|$) 7.40 ± 0.15 (SHR) and 7.26 ± 0.10 (WKY).

Figure 2 shows concentration-contraction relations for Ca$^{2+}$ in potassium depolarized aortic rings from SHR ($n = 6$) and WKY ($n = 10$). Arteries from SHR were less sensitive to the contractile effect of calcium than arteries from WKY, and this difference was statistically significant at all ECs except EC$_{10}$ and EC$_{30}$. EC$_{50}$ were ($-\log |M|$) WKY, 3.18 ± 0.03 and SHR, 2.76 ± 0.13 ($p<0.05$). The maximal forces obtained with Ca$^{2+}$ in SHR were not significantly different (inset).

The data of the calcium influx measured by the EGTA method appear in Figure 3. The basal influx was (in $\mu$M/kg ww/min) 27.1 ± 2.4 in WKY and 31.4 ± 1.5 in SHR. KCl 35 mM induced a significant increase in calcium influx in WKY and SHR. Potassium-chloride-promoted calcium influx was 42.3 ± 3.6 in WKY and 42.5 ± 2.2 in SHR (an increase of 56 and 35%, respectively). Nifedipine $10^{-10}$ M significantly decreased the calcium influx in WKY to 30.9 ± 3.3 ($p<0.05$), whereas no significant effect was detected in SHR (50.0 ± 2.4). When calcium influx was measured by the lanthanum method, the data obtained were essentially in accordance with those obtained with the EGTA method. Nifedipine $10^{-10}$ M did not produce any significant effect on calcium efflux measured under the same potassium-chloride concentration as Ca$^{2+}$ influx experiments.

Figure 4 is a simultaneous representation of the mechanical effect of nifedipine, expressed as relaxation, and its effect on calcium channels, measured as blockage of calcium influx. In SHR ($n = 9$), calcium influx blockage was dissociated from the mechanical effect, since relaxation was 100% with nifedipine concentrations of $10^{-10}$, $10^{-9}$, and $10^{-8}$ M, whereas influx blockages were 0, 26, and 80%, respectively (Figure 2A). In WKY ($n = 9$), the mechanical effect roughly paralleled the calcium influx blockage at all concentrations of nifedipine (Figure 2B).

Figure 5 (top) shows concentration-contraction curves in skinned arteries from NR ($n = 12$) and SHR ($n = 13$), with and without nifedipine $10^{-10}$ M. In the absence of nifedipine, arteries from SHR were less sensitive to the contractile effect of calcium than those from NR, resembling what has been previously found in non-skinned arteries (see Figure 2). pCa for EC$_{50}$ were NR, 6.20 ± 0.03 and SHR, 5.91 ± 0.05 ($p<0.05$). Nifedipine $10^{-10}$ M depressed the contractile response to calcium in NR but without statistical significance. In SHR, the response to the same concentration of nifedipine was more marked. This response was significant at all ECs calculated (pCa for EC$_{50}$ with...
The electromechanical coupling that connects membrane depolarization by high potassium with contraction involves calcium entry through voltage operated channels (VOCs). The VOCs are apparently the binding site for dihydropyridines, and the mechanism of action to produce relaxation seems to be the blockage of calcium influx. Under these experimental conditions, a greater sensitivity of hypertensive smooth muscle to nifedipine was detected. When another pharmacological intervention resulting in vasodilation (nitroglycerin) was used, no differences in sensitivity were detected in aortic smooth muscle from SHR to WKY. The hypertensive smooth muscle was more sensitive to nifedipine than to calcium. The same conclusion was previously drawn from experiments in which NR rats were used as controls. This precaution is necessary because extrapolation of data from the SHR model is known to be sensitive to the type of control used. The possibility of obtaining conflicting results from different controls was recently reconsidered.

The calcium sensitivity of smooth muscle from hypertensive animals has been the subject of several investigations. Hinke was the first to propose an enhanced calcium sensitivity in isolated perfused tail artery from deoxycorticosterone acetate (DOCA) hypertensive rats. Holloway and Bohr and Hansen and Bohr proposed a functional change in the vascular membrane itself as the mechanism involved in this phenomenon, rather than a secondary alteration to an increase in pressure. In 1978, however, Lederballe-Pedersen and coworkers reported a reduced sensitivity to calcium in aortas from SHR compared to NR. More recently a reduced sensitivity to extracellular calcium has been reported in different arteries of SHR. In part, the conflicting results may be attributed to the type of artery used, the way in which the channels were opened, the type of control used, the species differences, and the type of hypertension.

An alteration in calcium sensitivity in vessels from SHR could be located at several possible sites: 1) a change in membrane permeability to calcium; 2) changes in intracellular calcium handling mechanisms, and 3) a change in the calcium sensitivity of the contractile mechanisms. This last possibility can be tested by measuring calcium sensitivity in a functionally skinned vascular preparation devoid of plasma membrane and intracellular calcium pools. Under these controlled conditions, a decreased calcium sensitivity in hypertensive fibers was found (Figure 5). This is consistent with an alteration of the intracellular calcium handling mechanism. However, it cannot be denied that a change in membrane permeability could play an additional role in the altered calcium sensitivity of the hypertensive smooth muscle. The changes in calcium sensitivity were approximately twofold in both intact and skinned fibers (2.60 and 1.90, respectively). This suggests that the main mechanism determining the change in calcium sensitivity is intracellular.

Diminished calcium sensitivity of the contractile mechanism detected in skinned fibers from SHR can be caused by any of the steps involved in the myosin light chain kinase (MLCK) phosphatase system. A decrease in calmodulin concentration, a phosphorylation of MLCK by AMP-dependent protein kinase, an increase in phosphatase activity, or inhibitors that could interfere with the biological activity of calmodulin would result in a greater calcium requirement for a given level of myosin light chain phosphorylation and
contraction. Recent experiments by McMahon and Paul\textsuperscript{19} report decreased calcium sensitivity in intact aortas of DOCA hypertensive rats precontracted with potassium chloride. However, they could not detect an altered sensitivity when the aortas were skinned. These results contrast with those presented here, possibly because of the difference in models of hypertension used.

Data from this study on the smooth muscle from SHR provide information on 1) the lack of correlation between relaxation and calcium influx blockade; and 2) the action of the pharmacological compound in the skinned aorta.

The term "calcium antagonist" was first used by Fleckenstein.\textsuperscript{26} However, the term "calcium entry blockers" was subsequently proposed, based on the mechanisms of action involved in producing relaxation in vascular smooth muscle. A correlation between calcium influx blockade and relaxation was reported by van Breemen et al.\textsuperscript{16} and Godfraind et al.\textsuperscript{27} Our data on the smooth muscle from normotensive animals are in agreement with these previous reports. However, a lack of correlation between calcium influx blockade and relaxation was detected in hypertensive smooth muscle. In the SHR, the \( EC_{50} \) for relaxation was \( 10^{-13} \) M, but we were unable to detect calcium influx blockade up to \( 10^{-10} \) M of nifedipine. Calcium influx blockade was promoted in the completely relaxed state only after the dose was increased to \( 10^{-9} \) and \( 10^{-8} \) M, suggesting that a mechanism other than calcium influx blockade produces relaxation in hypertensive smooth muscle.

If the greater sensitivity of hypertensive smooth muscle to nifedipine is not followed by a greater calcium influx blockade, other mechanisms of relaxation should be considered. A greater calcium efflux promoted by the compound in the hypertensive smooth muscle could account for the enhanced relaxation. In normal smooth muscle, a lack of effect of nifedipine on calcium efflux was previously reported.\textsuperscript{28} Our data demonstrated no significant effect of nifedipine on calcium efflux in hypertensive smooth muscle.

The changes in calcium sensitivity produced by nifedipine \( 10^{-10} \) M were not statistically significant in the normotensive smooth muscle (either WKY or NR). However, they were significant at all calcium concen-
tions in the hypertensive smooth muscle. The change in calcium sensitivity brought about by this pharmacological intervention was about 1.94-fold in the hypertensive smooth muscle. This decrease in calcium sensitivity produced by nifedipine 10^{-10} M could account for relaxation of approximately 70% (Figure 5). The pharmacological interaction between the compound and some intracellular mechanisms leads to a decreased calcium sensitivity. This change in calcium sensitivity becomes significant in hypertensive smooth muscle. In this context, it should be mentioned that no effect from nifedipine was detected in saponin-skinned fibers from mesenteric artery of normal rabbits. 29

These data are consistent, then, with the hypothesis that nifedipine produces smooth muscle relaxation in SHR through mechanisms other than calcium influx blockade, and also with the possibility that this pharmacological intervention may result in a diminished formation of the Ca^{2+}-calmodulin-MLCK complex. This diminished formation could be the result of a binding of the drug to calmodulin, 25 a decrease in affinity of the Ca^{2+}-calmodulin complex for the light chain kinase, an increase in phosphatase activity, 26 or a direct action of the drug on the MLCK. In connection with this, the binding of dihydropyridines to calmodulin has already been reported but at doses higher than 10^{-10} M. 31,32 If the binding of dihydropyridines to calmodulin could play a role in the mechanism of relaxation, it would be possible to predict an enhanced effect if a deficit in calmodulin exists. A decrease in calmodulin concentration in smooth muscle from SHR was reported 26 and could explain not only the different calcium sensitivity but also the differences in pharmacological sensitivity to the compound, if we accept an anticalmodulin effect of the drug. In any case, in the light of the present results, it seems reasonable to reexamine the mechanism responsible for the vasodilator effect of NIF in hypertension and to consider the possibility that the term "calcium influx blocker" for NIF should be challenged, at least in SHR. However, since nifedipine is known to block mainly the VOCCs, and the physiological response of blood vessels depends to a large extent on receptor operated channels, extrapolation of our findings to the clinical situation should be made with caution.

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