Differences in Norepinephrine Activation and Diltiazem Inhibition of Calcium Channels in Isolated Rabbit Aorta and Mesenteric Resistance Vessels

Cynthia Cauvin, Scott Lukeman, John Cameron, Ok Hwang, and Cornelis van Breemen

SUMMARY. The mechanisms of norepinephrine stimulation of calcium ion entry in isolated rabbit aorta and mesenteric resistance vessels were studied through measurements of effects on calcium-45 influx, tension, and membrane potential. The resistance vessels were considerably less sensitive to norepinephrine than the aorta. The aorta exhibited complex dose-response curves for norepinephrine-stimulated calcium influx and contraction, whereas these were simple in the arterioles. Both vessels were depolarized with increasing concentrations of potassium. Norepinephrine did not depolarize the aorta, whereas it did depolarize the mesenteric resistance vessels. This result supports the contention that norepinephrine opens receptor-operated channels to induce calcium entry in the aorta, while it may activate potential sensitive calcium channels in the mesenteric resistance vessels. However, the maximum depolarization with norepinephrine (10^-4 M) in the arterioles was completely blocked by 10^-5 M diltiazem, whereas that induced by 80 mM potassium was unaltered by the diltiazem. Furthermore, 10^-1 M norepinephrine was able to stimulate virtually the same contraction and calcium influx in 80 mM potassium-depolarized arterioles as in normal polarized tissues. These results are consistent with norepinephrine opening of receptor-operated channels to allow calcium entry in the rabbit mesenteric resistance vessels. That the behavior of norepinephrine-activated channels in the aorta is more complex than in the arterioles is further illustrated by a dramatically decreasing sensitivity of norepinephrine-stimulated calcium influx to diltiazem with increasing norepinephrine in the aorta but not in the arterioles. We have hypothesized that the complexity of the norepinephrine-stimulated calcium influx in the aorta compared to the resistance vessels may be related to the substantial release of intracellular calcium in the former, such that, as release of intracellular calcium is increased, sensitivity of norepinephrine-activated channels to diltiazem decreases. (Circ Res 56: 822-828, 1985)

ORGANIC Ca++ antagonists demonstrate a remarkable degree of selectivity for particular tissues, even though their binding affinities to membranes isolated from these tissues are only minimally different (for reviews, see Flaim and Zelis, 1982; Merrill and Weiss, 1982; Cauvin et al., 1983; Janis and Triggle, 1984). For example, Towart (1981) has shown that nimodipine is 10,000 times more potent in relaxing the serotonin-contracted rabbit basilar artery than the saphenous artery. Similarly, diltiazem relieves norepinephrine (NE)-constricted rabbit mesenteric resistance vessels at concentrations which are five orders of magnitude lower than those needed to inhibit aortic norepinephrine contractions (Cauvin et al., 1984). To explain such selectivity in the light of uniform binding constants, it is necessary to compare mechanisms of Ca++ mobilization in arteries possessing different sensitivities to Ca++ antagonist-induced relaxation. For this purpose, we chose the rabbit aorta and mesenteric resistance vessels. The latter should be a particularly interesting model, since human resistance vessels are extremely important Ca++ antagonist targets, whereas very little is known about their Ca++ regulation.

Saída and van Breemen (1983) established that intracellular Ca++ release is far less sensitive to blockade by diltiazem and nisoldipine than is stimulated Ca++ entry. Furthermore, in most blood vessels, Ca++ antagonists more potently inhibit Ca++ entry stimulated by electrical and potassium-induced depolarization than that activated by pharmacological agonists. In the present study, we have investigated mechanisms of stimulated Ca++ entry and intracellular release in the aorta and resistance vessels by recording their contractile tension, Ca++ fluxes, and membrane potentials. Moreover, differences in these phenomena in the two vessels are discussed in relationship to differences in the pattern of diltiazem sensitivity of the Ca++ influx pathways in the two vessel types. (Cauvin et al., 1984). Parts of this work have been presented in preliminary reports (van Breemen et al., 1982; van Breemen et al., 1983; Cauvin and van Breemen, 1983; Lukeman and van Breemen, 1983).

Methods

New Zealand white rabbits were stunned and exsanguinated; the thoracic aorta and mesenteric vascular bed...
were then removed. Vascular tissues were cleaned of fat and surrounding tissue while incubated in warmed (37°C), oxygenated (100% O2), physiological salt solution (PSS) of the following composition (mM): NaCl (140), MgCl2 (1), CaCl2 (1.5), KCl (4.6), dextrose (10), HEPES buffer (5); pH = 7.4.

Contraction

For contraction studies, aortic rings were mounted as previously described (van Breezen et al., 1972). Isolated cylindrical segments (2 mm long) of fourth or fifth branches (=200 μm i.d.) of the superior mesenteric artery were mounted in a manner adapted from Bevan and Osher (1972) and Mulvany and Halpern (1977). Two 40-μm tungsten wires (Westinghouse, Inc.) were passed through the lumen of the isolated mesenteric resistance vessel. One wire was fastened with screws to a fixed tissue mount. The other wire was pulled taut by parallel hooks that were attached to a strain gauge force transducer (U-gage, Shinko Co, Ltd.), the position of which could be adjusted with a micromanipulator. A resting tension of 25 mg was placed on the tissues, which were allowed to equilibrate for 1 hour.

Concentration-response curves for NE were obtained in both vessel types by exchanging the bathing solution containing successively higher concentrations of NE. Contractions in Ca**+-free solution were obtained by omitting Ca** from the PSS and adding 2 mM EGTA; preincubation for 10 minutes in this solution was used for all contractions in Ca**+-free media.

**Ca Flux Measurements

Unidirectional Ca** influx measurements were made by a pulse labeling technique (Meisheir et al., 1981). After tissues were dissected and allowed to equilibrate in PSS for 2 hours, they were placed in Ca**+-containing media (either PSS or experimental media) for 90 seconds, then were washed for 40 minutes (resistance vessels) or 45 minutes (aortae) in ice cold, vigorously gassed with a calcium-free PSS solution containing 2 mM EGTA. Tissues then were blotted gently, weighed (to 0.01 mg), and placed in 5 mM EDTA solution overnight. A scintillation cocktail containing toluene and Triton X-100 was added and the tissues were sonicated for 2 hours, they were placed in 4°C Ca-containing media. Membrane potential measurements were then removed. Vascular tissues were cleaned of fat and surrounding tissue while incubated in warmed (37°C), oxygenated (100% O2), physiological salt solution (PSS) of the following composition (mM): NaCl (140), MgCl2 (1), CaCl2 (1.5), KCl (4.6), dextrose (10), HEPES buffer (5); pH = 7.4.

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Membrane Potential Measurements

To determine membrane potential, short (<1 cm) cylindrical segments of aorta were cut open and pinned, intimal surface upward, in an 80-mL Lucite tissue bath. Segments of the mesenteric vascular bed were mounted intact for electrophysiological recording from resistance vessels. The tissues were continuously superfused with warmed, oxygenated PSS. Transmembrane potentials were recorded with standard glass microelectrodes filled with 3 m KCl that had tip resistances in the range of 40–90 MΩ. The microelectrodes were connected to dual differential preamplifiers with input impedences of 109 MΩ (WPI, KS 700). Amplifier output was displayed on a calibrated oscilloscope (Tektronix) for visual determination of membrane potential. No potentials were accepted unless the following criteria were fulfilled: (1) upon initial impalement, potential changes were abrupt and subsequent drift was less than 10 mV; (2) membrane potential remained stable for at least 30 seconds; (3) upon retraction, the potential returned abruptly to baseline; (4) electrode resistance before and after an impalement was identical. Electrode resistance was also checked during each impalement, and when it increased dramatically, the electrode was removed from the cell and resistance was checked again. If the resistance remained elevated, the electrode was assumed to be plugged and was discarded. Also, care was taken to balance DC offset potentials for each electrode in order to account for microelectrode tip potentials.

At the end of the experiment, electrode tips were broken and tip potential was determined. These were always less than or equal to 6 mV. When feasible, electrode penetration was maintained during drug infusion. However, contraction induced by stimulatory agents often forced the microelectrode out of the cell. In either case, a series of at least 10 separate membrane potential determinations were carried out before and after each change in drug orionic concentration.

Drugs

The following drugs were utilized in this study: norepinephrine (l-arterenol bitartrate; Sigma) and diltiazem (Marion).

Statistics

Values are given as means ± se. To compare means, Student’s t-test for unpaired data was utilized. (P < 0.05 considered significant). Unless otherwise indicated, values indicate the number of animals used.

Results

The first three figures illustrate marked differences in the way NE activates the rabbit aorta and mesenteric resistance vessels. In the aorta, the threshold for contraction is 3 × 10^-9 M, but maximal contraction is not achieved until 10^-5 M NE (Fig. 1). The shallow concentration response curve has a Hill coefficient of 0.7, which may indicate negative cooperativity, more than one operative receptor, or multiple contributing processes. At least two processes appear to be operational, as indicated by the concentration-response curves for NE-stimulated 45Ca influx and the less NE-sensitive intracellular Ca++ release contractions (Fig. 1). The concentration response curve for NE-stimulated 45Ca influx appears to be biphasic, which may be suggestive of two receptor activated or two 45Ca influx processes. Receptor binding studies (Docherty et al., 1981), as well as our own previous work (Cauvin et al., 1982) indicate that only α1-adrenoceptors are involved in NE activation of this vessel. In contrast, the mesenteric resistance vessels present a much simpler picture with similar, monophase concentration-response curves for contraction and stimulated 45Ca influx and very little responsiveness in the absence of external Ca++ (Fig. 2). The resistance vessels respond to NE concentrations of 3 × 10^-7 through
**Figure 1.** Concentration-response curves for norepinephrine-induced contraction in normal physiological saline solution (PSS), stimulated calcium ($^{45}$Ca) influx, and contraction in calcium-free solution containing 2 mM EGTA in isolated rabbit aorta. Contraction values ($n = 4-5$) are expressed as means ± SEM. Calcium influx was calculated in $\mu$mol/kg wet weight. NE-stimulated $^{45}$Ca influx was calculated by subtracting the $^{45}$Ca influx in PSS from that obtained with NE. The maximum value of NE ($10^{-4}$ M)-stimulated $^{45}$Ca influx was $21 ± 2 \mu$mol $^{45}$Ca/kg in 90 seconds. NE stimulated $^{45}$Ca influx significantly at all concentrations equal to or greater than $10^{-4}$ M ($n = 4-5, 4-5$ tissues each). These data have been presented in a preliminary report (van Breemen et al., 1982).

$10^{-4}$ M in a manner consistent with a single activating process, namely, stimulation of Ca++ influx, with little to no release of intracellular Ca++.

We next examined the effects of NE on membrane potential ($E_m$) in the two artery types. Figure 3 illustrates that over the concentration range where NE activates the aorta, it causes no significant cell membrane depolarization. As expected, membrane depolarization was recorded in the aorta and mesenteric resistance vessels when the external K+ concentration was raised (Fig. 3). However, in contrast to the lack of NE depolarization in the aorta, it caused marked depolarization of the mesenteric resistance vessels. Figure 4 shows the voltage-tension curves for NE and K+-induced activation of the two vessels. The coupling of $E_m$ and tension changes in the aorta, and mesenteric resistance vessels activated by elevated K+ are fairly similar. The aorta begins to contract at a more polarized $E_m$ than do the resistance vessels. Tension develops from 20–100% of maximum with a 16-mV change in $E_m$ in the aorta compared to a 13-mV change in the mesenteric resistance vessels. The voltage-tension coupling relationship for depolarization induced by NE in the mesenteric resistance vessels has a markedly lower voltage threshold for tension development ($-53$ mV) as compared to that for K+-induced tension development ($-39$ mV). The voltage change for which tension develops from 20–100% of maximum is 14 mV, which is very similar to that observed with K+-induced depolarization and tension development.

From these data, it appeared likely that NE activated the rabbit aorta through pharmacomechanical coupling, while it activated the mesenteric resistance vessels through electromechanical coupling. To strengthen this conclusion, we performed a set of experiments similar to ones previously performed in rabbit aorta (Meisheri et al., 1981). In the rabbit...
aorta, 80 mM K* and 10−5 M NE were found to stimulate contraction and 45Ca influx in an additive manner. Since these two modes of activation represented maximal stimulation of their respective activation mechanisms, the additivity suggested that these activation mechanisms were distinct, as has been verified by the lack of depolarization by NE in the aorta (Fig. 3). We expected to find the opposite result in the mesenteric resistance vessels, since NE activation is associated with depolarization in these tissues (Fig. 3). We found, however, that after depolarization of the mesenteric resistance vessels with 80 mM K*, NE induced contraction and 45Ca influx to nearly the same extent as in normal polarized tissues (Fig. 5). The contraction by NE in high K* was 92 ± 5% of that obtained in normal PSS. The 45Ca influx stimulated by NE over 80 mM K* was 24 ± 8 μmol/kg wet tissue compared to 26 ± 5 μmol/kg stimulated by NE over basal levels in 4Ca-PSS. The stimulation of tension and 45Ca influx by NE in the 80 mM K*-depolarized tissues occurred in the absence of further depolarization by NE. These results led us to question whether NE-induced depolarization may be a secondary rather than primary event in excitation contraction coupling. To study this possibility, we determined the effects of the Ca++ entry blocker diltiazem (10−5 M) on NE and 80 mM K+-induced depolarization in these vessels. This concentration of diltiazem has been shown to inhibit completely the 45Ca influx stimulated by both these modes of activation in these vessels (Cauvin et al., 1984). The diltiazem had no significant effect on the resting Em or on the K* depolarization, but it completely inhibited the NE depolarization (Table 1).

The final comparison between the aorta and mesenteric resistance vessels was with regard to their patterns of diltiazem sensitivity of NE-stimulated 45Ca influxes. We reported previously that NE-stimulated 45Ca influx was more sensitive to diltiazem in the mesenteric resistance vessels for maximal activation (Cauvin et al., 1984). Figure 6 shows the IC50 for diltiazem inhibition of 45Ca influx stimulated by varying [NE]. The aorta is variable in its sensitivity to diltiazem with variable [NE]; i.e., as the level of NE activation increases, the diltiazem sensitivity decreases. In contrast, the mesenteric resistance vessels maintain their high sensitivity to diltiazem throughout the effective NE concentration range.

### Table 1

<table>
<thead>
<tr>
<th>Bathing medium</th>
<th>Membrane potential (mV)*</th>
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<tbody>
<tr>
<td>PSS + 10−5 M diltiazem</td>
<td>54 ± 3</td>
</tr>
<tr>
<td>PSS + 10−4 M norepinephrine</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>PSS + 10−4 M diltiazem + 10−4 M norepinephrine</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>80 mM K*</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>80 mM K* + 10−5 M diltiazem</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>80 mM K* + 10−4 M norepinephrine</td>
<td>19 ± 3</td>
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</tbody>
</table>

*Values are given as means ± SEM for at least five animals, with at least five determinations made per animal.
Discussion

The concept of pharmacomechanical coupling in smooth muscle has long been debated (Somlyo and Somlyo, 1968). Edman and Schild (1962) originally demonstrated that acetylcholine could cause a sustained contraction of uterine smooth muscle that was previously depolarized with a high K⁺ solution, and that the contraction was dependent upon extracellular Ca++. In 1964, Su et al. reported that perivascular nerve stimulation induced contraction of the rabbit pulmonary artery without depolarizing the smooth muscle membrane. Droogmans et al. (1977) later found that exogenously applied NE could produce maximal contraction of the rabbit ear artery without causing depolarization. These observations, along with the observation that the sustained phase of agonist-induced contraction of arteries generally is dependent upon extracellular Ca²⁺ (Bohr, 1963; Hink, 1964; Flaim and Zelis, 1982; Cauvin et al., 1983; Loutzenhiser et al., in press), led to the hypothesis that NE could stimulate Ca²⁺ entry into arterial smooth muscle through receptor-operated channels (ROC) rather than potential sensitive Ca²⁺ channels [PSC (Bolton, 1979; van Breemen et al., 1979)]. However, in many arterial preparations, agonists have been found to produce at least some degree of depolarization (Casteels et al., 1977; Haeusler, 1978; Hermsmeyer et al., 1981; Mulvany et al., 1982; Harder and Hermsmeyer, 1983). Nevertheless, three further lines of evidence which support the hypothesis that NE could stimulate Ca²⁺ entry through ROC in the isolated rabbit aorta are as follows: (1) ⁴⁵Ca influx stimulated by NE (10⁻⁵ M) is additive to that stimulated by 80 mM K⁺ (Meisheri et al., 1981); (2) ⁴⁵Ca influx induced by 80 mM K⁺ is selectively inhibited over that stimulated by 10⁻⁵ M NE when organic Ca⁺⁺ antagonists (Meisher et al., 1981; Cauvin et al., 1983) or dibutyryl cAMP (Meisher and van Breemen, 1982) are employed to inhibit Ca⁺⁺ entry; and (3) the ⁴⁵Ca influx stimulated by the new "Ca⁺⁺ channel agonist" Bay K8644 is additive to that stimulated by 10⁻⁵ M NE, but not to that stimulated by 80 mM K⁺ (Yamamoto et al., 1984). The final line of evidence that NE opens ROC in rabbit aorta is that it produces a tonic contraction that is dependent on Ca⁺⁺ influx without producing membrane depolarization (see Fig. 3). It could be argued that the cells near the medial surface do not represent all aortic smooth muscle cells, and, hence, that our EM measurements made from the intimal surface could have missed NE depolarization of cells near the adventitial surface. We feel that this is unlikely, since Mekata (1984) has shown that NE depolarized the muscle in the inner but not the outer regions of the rabbit carotid artery. Our results thus indicate that NE induces Ca⁺⁺ entry in the rabbit aorta and its consequent tonic contraction through pharmacomechanical coupling.

In the rabbit aorta, the concentration-response curve for NE-induced contraction is very shallow. Possible explanations for this observation are the presence of more than one type of α-adrenoceptor or the operation of multiple Ca⁺⁺ delivery systems. It has been suggested that α₁-adrenoreceptors activate intracellular Ca⁺⁺ release while α₂-adrenoreceptors are linked to Ca⁺⁺ channels (van Meel et al., 1981). This hypothesis was refuted, however, when it was found that the aortic NE contractions were initiated exclusively via α₁-adrenoreceptors (Cauvin et al., 1982). Nevertheless, it can be seen that the NE concentration ranges over which Ca⁺⁺ release and influx are stimulated are not identical, intracellular Ca⁺⁺ release being less sensitive to NE than Ca⁺⁺ influx. The concentration-response curve for NE-stimulated Ca⁺⁺ influx is also very shallow, with a second component of stimulated Ca⁺⁺ influx becoming apparent at NE concentrations which induce intracellular Ca⁺⁺ release. The absence of membrane depolarization during exposure of the aorta to NE rules out the possibility that the two Ca⁺⁺ influx components are due to sequential opening of ROC and PSC; however, different open states of the ROC are possible, and may be an explanation for the drastic change in their sensitivity in aorta to diltiazem as NE concentrations are varied (see Fig. 6).

Excitation-contraction coupling mechanisms for NE activation of the rabbit mesenteric resistance
vessels are apparently quite different from those in the aorta. Figure 2 shows for the full active NE concentration range that there is very little release of intracellular Ca++ in the rabbit mesenterial resistance vessels, whereas this phenomenon is substantial in the aorta. [This comparison for $10^{-5}$ M NE in the two vessels originally suggested this conclusion (Cauvin et al., 1984).] Moreover, NE depolarizes the plasmalemma in these vessels, while it has no effect on $E_M$ in the aorta (Fig. 3). Hence, whereas NE appears to induce contraction through pharmacomechanical coupling in the aorta, it is not completely clear how it does so in the resistance vessels. The electromechanical relationship for NE-induced depolarization and contraction of these vessels has the same slope as that for K+-induced depolarization and tension. The degree of $E_M$ change associated with 80% of the tension development for NE and K* is similar to that reported by others for peripheral but not cerebral arteries, where the $E_M$ changes are larger (see Hermansmeyer et al., 1981, for review). The threshold for tension development occurs at a more polarized $E_M$ for NE than for K*, suggesting that NE may modify the gating and conductance for PSC.

The latter hypothesis may also explain why addition of NE to vessels which were depolarized already with 80 mM K*, did not affect the $E_M$ (Table 1), but still increased tension and stimulated Ca influx to nearly the same extent as it did in normal polarized tissues (see Fig. 5). However, an alternative explanation for the increase in tension and Ca++ entry produced by NE under these conditions might be the opening of ionic channels similar to the ROC found in the rabbit aorta. Consistent with this possibility is the observation that diltiazem prevented NE- but not 80 mM K+-induced depolarization. This observation is similar to that of Ilo et al. (1978) who found that NE depolarization of the rabbit pulmonary artery was blocked by diltiazem. These results are unlikely to be due to specific a-adrenoceptor blockade, since diltiazem binds preferentially to Ca++ channels over a-adrenoceptors (Motulsky et al., 1983). The diltiazem may, however, inhibit both Ca++ and Na+ entry (Aaronson and Jones, 1983) in these vessels, thereby preventing NE depolarization. Bolton and Kitamura (1983) have provided evidence to support the hypothesis that activation of muscarinic receptors in guinea pig intestinal smooth muscle opens receptor-operated ionic channels, which can admit sufficient Ca++ to depolarize the cells and cause tension development. It is unclear at this time whether a similar conclusion can be reached in the rabbit mesenterial resistance vessels for NE activation.

One question that arises from this study is why NE depolarizes the mesenterial resistance vessels, but not the aorta. Droogmans et al. (1977) have suggested that the lack of NE-induced depolarization that they have found in the rabbit ear artery could be due to rectifying increases in other ion permeabilities (e.g. K*), such that the overall effect of NE on the membrane potential would not change. The lack of NE-induced membrane depolarization that we found in the rabbit aorta does not agree with the results of Mekata (1979), who found a 21-mV depolarization by $10^{-5}$ g/ml noradrenaline ($\sim 6 \times 10^{-5}$ M NE) in a PSS containing 2.5 mM Ca++. The greater [Ca++] used by Mekata would produce greater Ca++ entry in this vessel (Lodge and van Breemen, in press) than that in 1.5 mM Ca++ used in the present study. This greater Ca++ entry may be responsible for the depolarization seen by Mekata, if the Ca++ entry were not fully electrically compensated for by other ion fluxes.

The final question we examined was how the differences observed between the activating mechanisms of the two arteries might be related to their differences in diltiazem sensitivity. Ca influx stimulated by NE ($10^{-5}$ M) in the rabbit aorta has been shown to be far less sensitive to diltiazem inhibition than that in the mesenterial resistance vessels (Cauvin et al., 1984). It may be suggested that the depolarization by NE observed in the mesenterial resistance vessels but not in the aorta sensitizes these arteries to diltiazem; however, there are two indirect arguments against this explanation. One is the fact that, at low NE concentrations, the aorta does not depolarize, yet its NE-stimulated Ca++ influx at these low concentrations is almost as sensitive to diltiazem as that in the resistance vessels (Cauvin et al., 1984). The other is that the Ca influx induced by $10^{-5}$ M NE in the mesenterial resistance vessels is approximately 20 times more sensitive than that induced by 80 mM K*. An alternative explanation proposed for the differences in diltiazem sensitivity of the NE-activated Ca++ channels in the two vessels is that their sensitivity to diltiazem may be inversely related to the process of intracellular Ca++ release (Cauvin et al., 1984). It is clear that when comparing agonist-induced contractions and their sensitivity to Ca++ antagonists among various vessels, this sensitivity appears to be inversely related to the release of intracellular Ca++ by that agonist in different vessels (see Cauvin et al., 1983, for review). Furthermore, the NE-stimulated Ca++ influx in the rabbit aorta at high concentrations of NE is much less sensitive to diltiazem than at low concentrations of NE, and is less sensitive than in the resistance vessels at all NE concentrations (Fig. 6). Since this pattern is inversely correlated to the pattern of intracellular Ca++ release in these arteries, it is possible that the Ca++ release process may perturb the cell membrane in a manner which affects the a-adrenoceptor-activated Ca++ channels. In light of the clinical importance of Ca++ antagonist selectivity, we feel that the latter hypothesis deserves further investigation.

Our thanks to Libby Blasdel and Maria-Isabel Martinez for typing the manuscript.

This work was supported by National Institutes of Health Grants...
HL 29467 and HL 30412, and the American Heart Association, Florida Affiliate.

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Received January 25, 1984; accepted for publication February 28, 1985.

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INDEX TERMS: Norepinephrine • Membrane depolarization • Ca2+ channels • Ca2+ antagonists • Arteries and arterioles • Intracellular Ca2+ • Ca2+ influx

Circulation Research • Vol. 56, No. 6, June 1985


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Circ Res. 1985;56:822-828
doi: 10.1161/01.RES.56.6.822

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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