Calcium recruitment in renal vasculature: NE effects on blood flow and cytosolic calcium concentration

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Salomonsson, Max, and William J. Arendshorst. Calcium recruitment in renal vasculature: NE effects on blood flow and cytosolic calcium concentration. Am. J. Physiol. 276 (Renal Physiol. 45): F700–F710, 1999.—This study provides new information about the relative importance of Ca\(^{2+}\) mobilization and entry in the renal vascular response to adrenergic receptor activation. We measured renal blood flow (RBF) in Sprague-Dawley rats in vivo using electromagnetic flowmetry. We measured intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) in isolated afferent arterioles utilizing ratiometric photometry of fura-2 fluorescence. Renal arterial injection of NE produced a transient decrease in RBF. The response was attenuated, in a dose-dependent manner, up to ~50% by nifedipine, an antagonist of L-type Ca\(^{2+}\) channel. Inhibition of Ca\(^{2+}\) mobilization by 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester (TMB-8) inhibited the renal vascular effects of NE in a dose-dependent manner, with maximal blockade of ~80%. No additional attenuation was observed when nifedipine and TMB-8 were administered together. In microdissected afferent arterioles, norepinephrine (NE; 10\(^{-6}\) M) elicited an immediate square-shaped increase in [Ca\(^{2+}\)]\(i\), from 110 to 240 nM. This in vitro response was blocked by nifedipine (10\(^{-6}\) M) and TMB-8 (10\(^{-5}\) M) to a degree similar to that of the in vivo experiments. A nominally calcium-free solution blocked 80–90% of the [Ca\(^{2+}\)]\(i\) response to NE. The increased [Ca\(^{2+}\)]\(i\) induced by depolarization with medium containing 50 mM KCl was totally blocked by nifedipine. In contrast, TMB-8 had no effect. Our results indicate that both Ca\(^{2+}\) entry and mobilization play important roles in the renal vascular Ca\(^{2+}\) and contractile response to adrenergic receptor activation. The entry and mobilization mechanisms activated by NE may interact. That a calcium-free solution caused a larger inhibition of the NE effects on afferent arterioles than nifedipine suggests more than one Ca\(^{2+}\) entry pathway.

norepinephrine; adrenergic receptor; renal circulation; afferent arteriole; 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester; nifedipine; vascular smooth muscle

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vitro blood-perfused juxtamedullary nephron preparation suggest that calcium release from intracellular stores represents a substantial component of the afferent and efferent arterial response to NE (23).

The present study was designed to further evaluate mechanisms responsible for changes in [Ca\(^{2+}\)], and contraction in renal resistance vessels in response to stimulation of adrenoceptors. The pharmacological agents nifedipine and 3,4,5-trimethoxybenzoic acid-d8-(diethylamino)octyl ester (TMB-8) were used to determine the relative importance of calcium entry and mobilization, respectively. We assessed renal vascular reactivity in rats in vivo, using electromagnetic flowmetry to measure RBF. We measured the [Ca\(^{2+}\)] in vitro in isolated rat afferent arterioles using the ratiometric fluorescence of the indicator fura 2.

METHODS

RBF measurements. Experiments were performed on male Sprague-Dawley rats obtained from the Chapel Hill breeding colony. Body weight averaged 303 ± 12 g. The rats were fed standard rat laboratory chow and tap water ad libitum. Anesthesia was induced by intraperitoneal injection of pentobarbital sodium (65 mg/kg body wt), and the rats were placed on a servo-controlled heating table that maintained body temperature at 37°C. A tracheostomy was performed and a tracheal catheter was inserted to facilitate breathing. We cannulated the left carotid artery to monitor mean arterial pressure (Statham P23 Db transducer) and to obtain blood samples for hematocrit measurements. The right jugular vein was cannulated for administration of supplemental additions of pentobarbital sodium and isoncotic BSA (47 g/l) to replace losses associated with surgery (1.25 ml/100 g body wt). Thereafter, isoncotic albumin (10 µl/min) was infused continuously, for the duration of the experiment, to maintain hematocrit and plasma protein concentration at presurgical levels. We made midline and subcostal incisions to expose the abdominal aorta and the left kidney. A tapered and curved polyethylene PE-10 catheter was introduced into the left femoral artery and advanced through the abdominal aorta and ∼1 mm into the left renal artery (15, 43). We used a noncannulating electromagnetic flow probe (Carolina Medical Electronics) and placed around the left renal artery to measure RBF. We measured the RBF of pentobarbital sodium (65 mg/kg body wt), and the rats were placed in a 10-µl bolus of test agent into the renal artery infusion line. The RBF values presented are averaged for 5 s, during which blood flow to return to baseline values. The RBF values were normalized and expressed as a percentage of baseline values, which we calculated separately for each injection using the mean value observed during the 20-s time interval between introduction of NE and onset of the renal vascular response. The RBF values presented are averaged for 5 s, during which the blood flow response to NE was maximal. The recordings obtained using the antagonists and NE were analyzed in the same fashion.

Measurements of cytosolic calcium concentration. Glomeruli with attached afferent arterioles were microdissected from Sprague-Dawley rats (308 ± 14 g) of the Chapel Hill colony. Several thin slices (0.5–1 mm) were cut from the middle region of the kidney. The slices were transferred to a dish containing an ice-chilled physiological salt solution (PSS), with BSA (Sigma) added to give a final concentration of 0.5 g/dl. The PSS solution had the following composition: 135 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 1M g C l\(_2\), 2.5 mM CaCl\(_2\), 10 mM HEPES, and 5 mM d-glucose. We used sharpened forceps for the isolation procedure under Wild microscopic visualization (×12–100). An interlobular artery was localized at its origin from an arcuate artery, and a wedge-shaped segment, consisting of glomeruli, blood vessels, and tubular structures, was removed. To obtain a vessel, we carefully stripped the tubular structures away one by one using forceps. We cut a single afferent arteriole as close as possible to the bifurcation arising from an interlobular artery using a sharp blade. To obtain a homogeneous population of arterioles, we used the most superficial arterioles possible from the outer one-third of the cortex. These were also usually the longest. If no preparation was obtained during the first 60 min of dissection, the kidney was discarded.

After completion of the dissection procedure, a vessel was loaded with fura 2 acetoxyx methoxy ester (fura 2-AM) for 45–60 min in the dark, at room temperature, as previously described (26, 45). Fura 2-AM (Molecular Probes) was prepared as stock solution in DMSO (1 mM) and mixed with PSS to a final concentration of 2 µM and Pluronic F127 (0.01%; Molecular Probes) immediately before use. We then transferred the arteriole to a chamber containing PSS on the stage of an inverted microscope (Olympus model IX 70) using an Eppendorf micropipette. Thereafter, we gently aspirated the proximal end of the arteriole and the glomerulus into a concentric glass holding pipette using a syringe connected to

Data acquisition was performed as previously described (15, 43). Briefly, it consisted of an IBM-PC-compatible Pentium computer and an analog-to-digital converter (Data Translation). The flow probe was interfaced to the data acquisition system by an electromagnetic flowmeter (Carolina Medical Electronics model 500). A Hewlett-Packard model 8805 B carrier amplifier was used for the blood pressure sensor interface. The recordings were started when NE was introduced into the renal artery perfusion line and lasted for a period of 120 s, which was sufficient to allow blood flow to return to baseline values. The RBF values were normalized and expressed as a percentage of baseline values, which we calculated separately for each injection using the mean value observed during the 20-s time interval between introduction of NE and onset of the renal vascular response.

Experiments were performed on male Sprague-Dawley rats obtained from the Chapel Hill breeding colony. Body weight averaged 303 ± 6 g. The rats were fed standard rat laboratory chow and tap water ad libitum. Anesthesia was induced by intraperitoneal injection of pentobarbital sodium (65 mg/kg body wt), and the rats were placed on a servo-controlled heating table that maintained body temperature at 37°C. A tracheostomy was performed and a tracheal catheter was inserted to facilitate breathing. We cannulated the left carotid artery to monitor mean arterial pressure (Statham P23 Db transducer) and to obtain blood samples for hematocrit measurements. The right jugular vein was cannulated for administration of supplemental additions of pentobarbital sodium and isoncotic BSA (47 g/l) to replace losses associated with surgery (1.25 ml/100 g body wt). Thereafter, isoncotic albumin (10 µl/min) was infused continuously, for the duration of the experiment, to maintain hematocrit and plasma protein concentration at presurgical levels. We made midline and subcostal incisions to expose the abdominal aorta and the left kidney. A tapered and curved polyethylene PE-10 catheter was introduced into the left femoral artery and advanced through the abdominal aorta and ∼1 mm into the left renal artery (15, 43). We used a noncannulating electromagnetic flow probe (Carolina Medical Electronics) and placed around the left renal artery to measure RBF. Before starting the measurements, we allowed the animals to stabilize for 30–60 min after completion of the preparation.

The following drugs were used. NE (Winthrop Pharmaceuticals) was dissolved in water. Nifedipine (Biomol) was mixed as stock solution (9 mg/ml) in DMSO and diluted in water immediately before use. This drug was used to reversibly inhibit calcium entry through L-type calcium channels. TMB-8 (Biomol) was diluted in saline and used to inhibit cellular mobilization by interfering with IP\(_3\)-induced calcium release from the endoplasmic reticulum (24, 40).

A Cheminert sample injection valve was used to introduce a 10-µl bolus of test agent into the renal artery infusion line (15, 43). One minute before administration of NE, the rate of renal artery infusion was increased to 144 µl/min. This rate of infusion allowed administration of the bolus volume within 5 s. After recovery of RBF to baseline levels (usually within 1 min), the infusion rate was returned to 5 µl/min. Nifedipine was administered as a bolus together with NE, whereas TMB-8 was continuously infused for 2 min before NE injection. In each rat, doses between 10 and 40 ng of NE were injected into the renal artery to find a dose that produced a 35–50% reduction in RBF. That particular dose was then used throughout a given experiment. The time interval between successive injections was 5–10 min. Preliminary studies confirmed earlier observations that vehicle infusion, continuous infusion of TMB-8, or bolus injection of nifedipine did not affect basal RBF or arterial blood pressure (15, 43). Furthermore, pilot studies established that repetitive administration of NE did not change basal steady-state RBF and arterial blood pressure during the course of an experiment.

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the back of the pipettes to generate negative pressure. For measurements of \([\text{Ca}^{2+}]\), the artery was centered in the optical field of a 40x quartz oil-immersion objective. The preparation was visualized by video camera (Sony) and monitor. Variable shutters were adjusted to center an artery in the sampling window. This arrangement made possible continuous control of the position of the preparation throughout an experiment. The artery was excited alternatively with ultraviolet light of 340 and 380 nm wavelength from a dual excitation wavelength DeltaScan equipped with dual monochromators and a light pathway chopper (Photon Technology International, South Brunswick, NJ). Fluorescent light was detected by a photometer after passing signals through a 510-nm bandpass filter. The fluorescence signal intensity was processed and stored by an IBM-compatible Pentium computer and Felix software (Photon Technology International). Intracellular \([\text{Ca}^{2+}]\) was calculated based on the ratio at 340/380 nm, according to the equation described by Grynkiewicz et al. (18): 
\[
[\text{Ca}^{2+}]_i = K_d \cdot \frac{(R - R_{\text{min}})/(R_{\text{max}} - R)) \cdot (S_f/S_b)}{S_f/S_b}
\]
where \(K_d\) is the dissociation constant of fura 2 for calcium; \(S_f\) and \(S_b\) are the 380-nm fluorescence at saturating and zero calcium concentrations, respectively; and \(R_{\text{min}}\) and \(R_{\text{max}}\) are values of \(R\) (fluorescence ratio 340/380) at low and at saturating calcium concentration, respectively. Values for \(K_d\), \(R_{\text{min}}, R_{\text{max}}, S_f,\) and \(S_b\) were determined in vitro, as previously described (26, 27, 45).

The volume of fluid in the experimental chamber was maintained at a constant level with the use of a vacuum suction system. The experiments were performed at room temperature (25–28°C). The experimental solutions were added by a large volume that allowed total exchange of the composition in the experimental chamber several times. We obtained a nominally calcium-free solution by adding 2 mM EGTA to PSS and replacing CaCl\(_2\) with NaCl. In the 50 mM KCl solution (K50), 45 mM of NaCl were replaced with an equal concentration of KCl. Pilot studies revealed that 10\(^{-6}\) M of NE elicited one-half of maximal response in \([\text{Ca}^{2+}]_i\); this concentration was used throughout the studies of isolated vessels. Nifedipine was dissolved in DMSO and diluted in PSS and K50 to a final concentration of 10\(^{-6}\) M. TMB-8 was added to the same solutions to yield a final concentration of 10\(^{-6}\) M.

In the in vitro experiments were conducted as follows. Initially, we tested the viability of each vascular preparation by adding a short pulse (50 s) of NE to the bath solution to provide a final concentration of 10\(^{-6}\) M. If there was no immediate \([\text{Ca}^{2+}]_i\) response, the preparation was discarded. Inhibitory agents were added before or after NE stimulation. In the pretreatment series, arterioles were exposed to nifedipine (50 s), calcium-free medium (50 s), or TMB-8 (120 s) before the addition of NE or K50, in the presence of the pretreatment solution (50 s). The \([\text{Ca}^{2+}]_i\) response obtained during inhibitor treatment was compared with the response when NE or K50 was added directly to the PSS solution in the absence of the inhibitor. The mean \([\text{Ca}^{2+}]_i\) values during the control period were obtained between 10 and 15 s before the addition of NE or K50. Likewise, \([\text{Ca}^{2+}]_i\) was measured between 10–15 s (initial peak response) and between 30–35 s (sustained plateau phase) after the stimulation was initiated. The experiments were performed in random order to establish reversibility and to exclude possible prolonged action of a particular pretreatment. In the poststimulation experiments, responses to NE or K50 were recorded for 50 s before a 50-s challenge with nifedipine, calcium-free medium, or TMB-8 in the presence of NE or K50. For this series, we present the plateau values of \([\text{Ca}^{2+}]_i\) before and after inhibition.

Statistical analysis. Data are presented as means ± SE. The Sigma Stat (SPSS, Chicago, IL) and Statistica (StatSoft Scandinavia) software were used for statistical analysis. Statistical significance was evaluated by ANOVA for repeated measurements and Newman-Keuls test. Student's t-test was used for paired observations. \(P < 0.05\) was considered significant.

RESULTS

Measurements of RBF. In 13 Sprague-Dawley rats, RBF during euvoletic control conditions averaged 5.1 ± 0.3 ml·min\(^{-1}\)·g kidney wt\(^{-1}\). Arterial blood pressure and hematocrit averaged 118 ± 2 mmHg and 48 ± 1%, respectively. In blood flow experiments, we used the dihydropyridine agent nifedipine to determine the involvement of voltage-sensitive calcium channels in the renal vascular response to adrenoceptor activation with NE. Figure 1A shows that bolus injection of NE produced a transient 32 ± 3% maximum reduction in RBF (n = 12; \(P < 0.001\) vs. basal RBF) normalized to an NE effect of 100%. Concomitant administration of
nifedipine (150, 750, and 1,500 ng) with NE attenuated the vasoconstrictor effect of NE. The lowest tested dose of nifedipine (150 ng) buffered the NE-induced decrease in RBF to 24 ± 3% of basal flow (P < 0.002 vs. NE). Nifedipine at 750 ng further diminished the response to NE to 17 ± 2% of basal flow (P < 0.02 vs. 150 ng). No additional inhibitory effect was observed when the nifedipine dose was increased to 1,500 ng (18 ± 2% of basal flow). Thus the strongest antagonistic action of nifedipine reached a plateau at ~50% inhibition of the NE-induced renal vasoconstriction. There was a complete recovery of the inhibitory effects of all doses of nifedipine within the standard time interval (>5 min) between successive injections.

In another series of studies, we evaluated the role of phospholipase C and IP$_3$-induced calcium mobilization from the sarcoplasmic reticulum in the renal vascular response to activation of adrenoreceptors by NE. Administration of NE during the control period produced a transient RBF reduction of 37 ± 3% of the basal value (n = 8; P < 0.001). Thereafter, TMB-8 was infused into the renal artery for 2 min before the bolus injection of NE, for the purpose of inhibiting calcium release from intracellular stores. These results, summarized in Fig. 1B, show that TMB-8 infusion, at a rate of 15 µg/min, reduced the NE response to 29 ± 4% of basal RBF (P < 0.04 vs. NE alone). The NE-induced reduction in RBF was further reduced to 14 ± 2% of basal flow by pretreatment with 30 µg/min of TMB-8 (P < 0.001 vs. 15 µg/min). Infusion of TMB-8 at 60 µg/min reduced the NE response to 7 ± 1% of basal RBF (P < 0.03 vs. basal RBF), a value not significantly different from the value obtained on TMB-8 infusion with 30 µg/min. These observations indicate that intracellular calcium mobilization plays a major role in the adrenoceptor-mediated renal vasoconstriction.

We attempted to gain insight into whether these two calcium recruitment mechanisms act independently of each other or they share or interact in a common signaling pathway. Based on the experiments presented earlier (see above), we selected doses of nifedipine and TMB-8 that exerted near maximum inhibition. During the control period in these animals, NE reduced RBF by 50 ± 5% (n = 6; P < 0.001). Nifedipine (750 ng) blunted the NE response to 29 ± 4% of basal flow (P < 0.001 vs. NE alone), reducing the control response by 42%. Pretreatment with TMB-8 (30 µg/min) reduced the NE effect to 17 ± 4% of basal flow, which represents a 66% reduction of the response in the absence of an inhibitor (P < 0.001 vs. NE). Simultaneous administration of these two inhibitors reduced the NE-induced decrease in RBF to 12 ± 2% of basal values. This level of inhibition (76%) is slightly larger than the value obtained with TMB-8 alone, but the difference is not statistically significant. The data shown in Fig. 1C do not support the notion that the two calcium mechanisms are additive and independent. Rather, the data suggest the interesting possibility that these major pathways interact or share a common event in their signaling pathways after adrenoceptor activation.

Measurements of afferent arteriolar [Ca$^{2+}$]. In 16 afferent arterioles from 14 rats, the [Ca$^{2+}$], averaged 110 ± 11 nM. Preliminary studies demonstrated that 10$^{-6}$ M of NE elicited one-half of maximal response in [Ca$^{2+}$]; this concentration was used in all of our studies of microdissected vessels. Addition of NE (10$^{-6}$ M) to the bath caused an abrupt step increase in vascular smooth muscle [Ca$^{2+}$] (Fig. 2, A and B). The response consisted of a sharp rise that was sustained at a near-maximal plateau level. In a few vessels, however, the transient initial peak was appreciably larger than the sustained plateau. To analyze the responses, we selected the timepoints of 10–15 s and 30–35 s as being representative of initial and sustained phases of the calcium response pattern, respectively. The time interval of 10–15 s captured early responses that were relatively stable, excluding stimulation artifacts associated with solution changes and large, very transient spikes, which were rare. The time period of 30–35 s was sufficient to obtain values indicative of sustained increases in [Ca$^{2+}$]. Preliminary studies established that the response at 30 s was commonly maintained for up to 4 min. Visible contraction of the arteriole frequently
correlated temporally with the increase in $[\text{Ca}^{2+}]_i$ observed on the video monitor.

In a series of paired experiments on individual afferent arterioles, the relative roles of calcium entry and mobilization, in response to activation of adrenoceptors, were investigated. We assessed the extent to which pretreatment with 2 mM EGTA (to render the medium nominally calcium-free), dihydropyridine nifedipine ($10^{-6}$ M), or TMB-8 ($10^{-5}$ M) affected the $[\text{Ca}^{2+}]_i$ response to NE. Either the calcium-free solution or nifedipine was added 50 s before NE stimulation (Fig. 2A). The intracellularly acting agent TMB-8 was added 120 s before administration of NE to allow enough time to gain access to cytosolic sites. The NE response obtained in the presence of a pretreatment solution was compared in a paired manner with the response obtained with NE alone, in the absence of an inhibitor. Control and recovery responses were assessed either before or after the recording of the NE response during the experimental period involving treatment.

To investigate the importance of calcium entry from the extracellular compartment, we assessed the effect of pretreatment with nominally calcium-free bath solution on the NE-induced calcium response. In these preparations, administration of NE in the control period increased $[\text{Ca}^{2+}]_i$ from $94 \pm 14$ to $217 \pm 26$ nM at 10–15 s (255%) and to $216 \pm 27$ nM at 30–35 s (244%; $P < 0.001$). After short-term exposure to the calcium-free solution, baseline levels of $[\text{Ca}^{2+}]_i$ decreased from $100 \pm 17$ to $85 \pm 15$ nM ($P < 0.05$). NE challenge in the presence of the calcium-free solution caused a small but significant (40%) increase in $[\text{Ca}^{2+}]_i$, at 10–15 s only (from $85 \pm 15$ to $110 \pm 18$ nM; $P < 0.01$, $n = 7$). After continued NE stimulation for 30–35 s, the change in $[\text{Ca}^{2+}]_i$ decayed somewhat, such that the late plateau phase did not differ from control $[\text{Ca}^{2+}]_i$ ($98 \pm 18$ vs. $85 \pm 15$ nM; Fig. 3C). Thus the stimulatory effect of NE was significantly attenuated when tested in nominally calcium-free solution ($P < 0.01$).

To evaluate the role of L-type calcium channels in the calcium entry after stimulation with NE, we used the dihydropyridine nifedipine. Before treatment with nifedipine, NE caused almost a 100% increase in $[\text{Ca}^{2+}]_i$, from $141 \pm 24$ to $251 \pm 32$ nM and $246 \pm 30$ nM at 10–15 s and 30–35 s, respectively ($P < 0.001$; $n = 9$). Pretreatment with nifedipine for 50 s did not affect baseline $[\text{Ca}^{2+}]_i$, but significantly attenuated the response to NE both at 10–15 s and at 30–35 s ($P < 0.05$). An individual response is shown in Fig. 2A. Group averages are presented in Fig. 3A. The percentage increase in $[\text{Ca}^{2+}]_i$ was close to 35%. The absolute values increased from $132 \pm 17$ to $176 \pm 24$ nM and to $175 \pm 25$ nM for the early and late responses, respectively ($P < 0.01$ vs. nifedipine).

In other studies, we tested the inhibitor effect of TMB-8. In the control period before drug treatment, NE elicited 135% increments in $[\text{Ca}^{2+}]_i$, from $101 \pm 15$ to $216 \pm 31$ nM at 10–15 s and to $217 \pm 30$ nM at 30–35 s ($P < 0.001$). Two minutes of exposure to TMB-8 did not affect baseline $[\text{Ca}^{2+}]_i$, but did significantly attenuate the $[\text{Ca}^{2+}]_i$ response to NE, both at 10–15 s and 30–35 s ($P < 0.05$). As Fig. 3B shows, $[\text{Ca}^{2+}]_i$ rose from $103 \pm 18$ to $130 \pm 27$ nM at 10–15 s and to $124 \pm 23$ nM at 30–35 s ($P < 0.05$ for both; $n = 7$).

We also determined the blocking effects of a calcium-free medium, nifedipine, or TMB-8 on the sustained plateau increase in $[\text{Ca}^{2+}]_i$, during continued adrenoceptor stimulation. These experiments enabled us to independently compare the effects of these agents on the initial rise and the sustained increase in $[\text{Ca}^{2+}]_i$, and thus evaluate events taking place during a maintained plateau phase. The poststimulation experiments are summarized as percent of baseline in Fig. 4.

In some preparations, we investigated the short-term effects of a calcium-free solution, applying EGTA after 50 s of NE stimulation. The results clearly show that the NE-induced $[\text{Ca}^{2+}]_i$ plateau was completely depen-
sudden decrease in [Ca$^{2+}$]. Antagonism of L-type calcium channels produced a 90% rise in [Ca$^{2+}$]. The group means are shown in Fig. 4. In the absence of the drug (n = 4), the [Ca$^{2+}$] increased from 111 ± 6% to 118 ± 6% (P < 0.001). The response to K50 was square-shaped, with the [Ca$^{2+}$] stable at 194 ± 49 nM at 30–35 s (P < 0.02 vs. control). When the preparations were exposed to nifedipine for 50 s before and during the addition of K50, there was no [Ca$^{2+}$] response to the depolarizing treatment. The basal [Ca$^{2+}$] value in the presence of nifedipine was 106 ± 19 nM and did not differ after K50 administration (110 ± 20 nM and 108 ± 18 nM) at 10–15 s and 30–35 s, respectively (Fig. 5A). This observation was confirmed in posttreatment experiments. The K50-induced elevation in [Ca$^{2+}$], from 103 ± 27 to 156 ± 28 nM (164 ± 35%; n = 5, P < 0.03) was totally abolished by the addition of nifedipine as [Ca$^{2+}$], returned to control levels (109 ± 19 nM or 110 ± 6%). Because large amounts of TMB-8 may counteract the effect of depolarization on vascular tissue (1), we performed additional experiments to rule out the possibility that the blocking effect of this substance, both in such channels, we performed experiments in which smooth muscle cell membranes were depolarized using a solution high in potassium (K50). The results establish that K50 initiated an 85% increase in [Ca$^{2+}$], from a basal value of 110 ± 20 to 196 ± 47 nM at 10–15 s (n = 7; P < 0.04). The response to K50 was square-shaped, with the [Ca$^{2+}$] stable at 194 ± 49 nM at 30–35 s (P < 0.02 vs. control). When the preparations were exposed to nifedipine for 50 s before and during the addition of K50, there was no [Ca$^{2+}$] response to the depolarizing treatment. The basal [Ca$^{2+}$] value in the presence of nifedipine was 106 ± 19 nM and did not differ after K50 administration (110 ± 20 nM and 108 ± 18 nM) at 10–15 s and 30–35 s, respectively (Fig. 5A). This observation was confirmed in posttreatment experiments. The K50-induced elevation in [Ca$^{2+}$], from 103 ± 27 to 156 ± 28 nM (164 ± 35%; n = 5, P < 0.03) was totally abolished by the addition of nifedipine as [Ca$^{2+}$], returned to control levels (109 ± 19 nM or 110 ± 6%). Because large amounts of TMB-8 may counteract the effect of depolarization on vascular tissue (1), we performed additional experiments to rule out the possibility that the blocking effect of this substance, both in

![Graph](image-url) Fig. 4. Group averages for cytosolic calcium concentration in afferent arterioles during control conditions, after NE stimulation, and then after stimulatory inhibition with nifedipine, TMB-8, or a calcium-free medium during continued exposure to NE. Values are means ± SE. Each group consisted of 4 or more arterioles. **P < 0.05 vs. baseline; ##P < 0.05 vs. NE before exposure to inhibitor.

As the individual experiment in Fig. 2B shows, the response to NE was attenuated by subsequent administration of nifedipine. The group averages shown in Fig. 4 indicate that NE caused a rise in [Ca$^{2+}$], to 180% of baseline, increasing from 106 ± 11 nM to the sustained plateau level of 179 ± 15 nM (P < 0.001; n = 12). Antagonism of L-type calcium channels produced a sudden decrease in [Ca$^{2+}$], to a new plateau level that averaged 134 ± 11 nM or 32% above control. The post-nifedipine level was significantly different, both from the control value and the plateau level in the absence of the drug (P < 0.02 and 0.001, respectively). These data indicate that a substantial part of the maintained increase in adrenoceptor activation of [Ca$^{2+}$] is dependent on dihydropyridine-sensitive calcium channels and correlates well with the results obtained in the pretreatment experiments.

In the same manner, we investigated the effect of TMB-8 on the sustained NE-induced [Ca$^{2+}$] plateau. The group means are shown in Fig. 4. In the absence of the inhibitor, NE elicited a 90% rise in [Ca$^{2+}$], from 89 ± 7 to 163 ± 21 nM (n = 5). On the addition of TMB-8 + NE, the [Ca$^{2+}$] response to NE significantly decreased to 111 ± 8 nM; the small, 28% increase did not differ statistically from control (P > 0.2).

The single vessel experiments utilizing nifedipine and the nominally calcium-free solution indicate that calcium entry from the extracellular space, presumably through voltage-sensitive channels, plays an important role in the renal vascular response to activation of renal adrenoceptors. To further establish the existence of...
vivo and in vitro, was due to action on voltage-sensitive calcium entry. Pretreatment with TMB-8 had no effect on the K50-induced elevation of [Ca\(^{2+}\)]. In the absence of TMB-8, K50 elicited an increase in [Ca\(^{2+}\)], from 88 ± 7 to 150 ± 26 nM at 10–15 s and 151 ± 24 nM at 30–35 s (n = 5, P < 0.03). After 2 min of TMB-8 pretreatment, the response to K50 was similar to the control response. In the presence of TMB-8, the corresponding K50 increased [Ca\(^{2+}\)], from 86 ± 9 to 129 ± 13 nM at 10–15 s and 136 ± 24 nM at 30–35 s (P < 0.01 vs. TMB-8), which was not significantly different from the control values (Fig. 5B). Also, in poststimulation experiments, we found that TMB-8 did not significantly attenuate the effect of sustained depolarization. The K50-induced [Ca\(^{2+}\)], plateau of 195 ± 54% vs. control was maintained at 183 ± 45% on addition of TMB-8 (P < 0.03 vs. baseline value). These results indicate that it is unlikely that the substantial attenuating effect of TMB-8 seen on adrenoceptor-induced renal vascular response is due to a major action on voltage-dependent calcium entry.

**DISCUSSION**

The present study investigated the cellular mechanisms mediating adrenoceptor-induced activation of smooth muscle cells of resistance vessels in the renal microcirculation. One aim was to determine the relative importance of entry of extracellular Ca\(^{2+}\) and mobilization of Ca\(^{2+}\) from intracellular stores in afferent arterioles in vivo and in vitro after activation of renal vascular adrenoceptors with NE. To this end, RBF was quantitated, in combination with measurements of [Ca\(^{2+}\)], in individual afferent arterioles isolated from Sprague-Dawley rats. An important aspect of this work is the comprehensive evaluation of vascular responses to a naturally occurring catecholamine based on hemodynamic assessment of contractile effects in vivo and fura 2-photometry determinations of calcium signaling in single microdissected afferent arterioles. Initial experiments established that injection of NE into the renal artery produced transient renal vasoconstriction. The magnitude of the constriction response was dose dependent, and total ischemia was noted with high doses of NE. To standardize the magnitude of NE-induced changes in renal vascular resistance, we adjusted the dose of NE in each animal to produce a 30–50% reduction in RBF, and then that particular dose was kept constant during subsequent administration of pharmacological inhibitors. Earlier studies have established that NE is a potent constrictor of the renal vasculature (29, 32, 33, 39, 46).

Isolated vascular segments exposed to NE in vitro contract whether NE is applied to the luminal or the basolateral side of the vessel (2, 7, 13, 22, 28, 29, 55). Other studies have reported effects of NE on [Ca\(^{2+}\)] in isolated renal vessels (29, 45, 54). However, there is a paucity of information concerning the intracellular signaling mechanisms that mediate adrenoceptor-induced action on renal resistance vessels.

Utilizing a whole kidney model, the Chapel Hill laboratory had previously shown that the vasoconstrictor actions of ANG II and vasopressin involve a combination of calcium entry that is antagonized by a calcium channel blocker and calcium mobilization sensitive to antagonists of an IP\(_3\) receptor (15, 43). About 50% of the in vivo constrictor response is mediated by an L-type calcium channel sensitive to the dihydropyridine nifedipine. Roughly the same magnitude of the vasoconstriction derives from calcium mobilization from TMB-8- or heparin-sensitive intracellular stores such as the sarcoplasmic reticulum, which is stimulated mainly by IP\(_3\). Furthermore, these two basic mechanisms appear to operate independently of each other as the actions of the two classes of inhibitors were additive, albeit not complete.

In the present study, we found that the NE-induced renal vasoconstriction was attenuated in a dose-dependent manner by administration of the dihydropyridine nifedipine. The two highest doses of nifedipine produced ~50% inhibition of the NE-induced renal vasoconstriction, suggesting a maximal effect. Nifedipine reduced NE-induced vasoconstriction from a control of 32% decrease in RBF to 17% of basal flow. Therefore, about one-half of the renal vascular response to adrenoceptor activation with NE is dependent on calcium entry via dihydropyridine-sensitive channels, which is consistent with the current literature. The non-dihydropyridine calcium channel blocker verapamil antagonizes the renal vasoconstriction elicited by NE infusion in dogs, but surprisingly the vasoconstriction produced by renal nerve stimulation in the same study was unaffected by verapamil (39). A recent study reports that cilnidipine, a blocker of L- and N-type calcium channels, inhibits renal vasoconstriction produced by NE, ANG II, and renal nerve stimulation in anesthetized dogs (47). In addition to blocking calcium entry into vascular smooth muscle cells, cilnidipine reduced the blood flow responses to increased nerve activity by attenuating NE release from nerve endings. Intrarenal arterial infusion of cilnidipine (0.3 µg·kg\(^{-1}\)·min\(^{-1}\)) produced a 50% attenuation of the NE-induced decrease in RBF. It is noteworthy that the effects of ANG II tended to be more dependent on calcium entry as the calcium channel blocker attenuated ANG II-induced vasoconstriction by 70% (47). This notion is supported by another report showing that verapamil exerts weaker blockade on the renal vascular response to NE than to ANG II in the dog (39).

Based on studies of the isolated, saline-perfused, chronically hydrenephrotic rat kidney, vasoconstriction elicited by NE and ANG II is almost completely reversed by the administration of 10\(^{-4}\) M of three different dihydropyridines (21). During maximal vasoconstriction in the basilar state, the renal vasoconstriction produced by NE in extirpated, saline-perfused, normal rat kidneys is markedly attenuated by verapamil or diltiazem, highlighting a strong dependence on voltage-sensitive calcium channels in this preparation, with abnormal vascular resistance and hemodynamics (46). Moreover, it should be noted that a rather high concentration of the calcium channel blockers (5 µM) was utilized in this study, so it is difficult to exclude a
non-specific action of the pharmacological agents. In contrast, other studies of isolated, saline-perfused rat kidneys report that the dihydropyridines nisoldipine and diltiazem have a stronger inhibitory effect on the NE actions on glomerular filtration rate (GFR) than on the NE-induced decrease in renal plasma flow (30, 32, 33). These whole kidney results were interpreted as suggesting a preferential action of the calcium channel antagonists on preglo-merular resistance vessels. We observed that nifedipine markedly attenuated the NE-induced decrease in RBF in vivo. Thus our hemody-namic studies, conducted under physiological conditions, contrast with these results derived from isolated kidneys, perfused artificially with a saline, non-blood solution.

In the in vitro setting, we found that NE stimulation produced an abrupt increase in [Ca$^{2+}$]i in isolated afferent arterioles. The shape of the response was consistent, characterized by a square-shaped increase in [Ca$^{2+}$]i, as immediate changes recorded 10–15 s poststimulation were maintained at 30–35 s (Figs. 2 and 3). Increases in [Ca$^{2+}$]i in vascular smooth muscle cells are normally associated with muscle contractile force and constriction (4). Using confocal microscopy to estimate changes in [Ca$^{2+}$]i in preglo-merular vessels in juxtamedullary nephrons of the rat kidney, NE-induced changes in afferent arteriolar [Ca$^{2+}$]i were found to be tightly correlated with changes in vessel diameter (54). The temporal response reported by these investigators was a rapid increase in [Ca$^{2+}$]i, followed by oscillating waves of elevated [Ca$^{2+}$]i, between 120% and 150% of baseline values, with a frequency of 0.1–0.2 Hz, that were synchronized with vessel contraction. The reason for the difference in the sustained changes in [Ca$^{2+}$]i may reflect variations in preparations, experimental design, anatomical location in the renal cortex, or signaling pathways in vascular smooth muscle cells and/or endothelial cells. It should be noted that the degree of inhibition we observed with in vitro nifedipine, applied either before or after NE stimulation in isolated vessel segments, was similar to that observed in the animal blood flow studies. In one set of experiments, nifedipine antagonized about one-half of the effect due to NE, providing strong evidence for a role of dihydropyridine-sensitive L-type channels in afferent arteriolar responses, as they participate in the regulation of renal hemodynamics by the sympathetic nervous system. The fact that both pre- and poststimulation application of nifedipine attenuates the [Ca$^{2+}$]i response highlights the importance of dihydropyridine-sensitive calcium channels in both the initial and sustained phases. Evidence for the existence of voltage-gated L-type calcium channels is also provided by our observation of increased [Ca$^{2+}$]i during stimulation with a high-potassium medium. This view is reinforced by the finding that nifedipine completely blocks the [Ca$^{2+}$]i response to depolarization induced by a high-potassium solution. This observation also excludes possible non-specific effects of the potassium gradient and of the calcium channel antagonist at the employed concentration and effectiveness of the dihydropyridine to block all activated calcium entry channels.

The [Ca$^{2+}$]i response to NE was reduced in the absence of extracellular calcium. Both the immediate peak and the sustained plateau phases were reduced by short-term (50 s) pretreatment with a nominally calcium-free medium, resulting from the removal of calcium and addition of 2 mM EGTA. The peak response to NE (10–15 s) was attenuated by 75% on the average, and the usual prolonged elevation (30–50 s) was reduced to a [Ca$^{2+}$]i level not significantly different from the level observed before the administration of NE. The fact that the nominally calcium-free medium caused a more pronounced inhibition of NE-induced [Ca$^{2+}$]i increases than nifedipine implicates a second calcium entry pathway, one that is insensitive to the dihydropyridine class of agents. Previous studies provide some support for the participation of more than one calcium entry pathway (17, 35, 44). A dependence of the hemodynamic response to NE on extracellular calcium concentra-tion was reported for the isolated perfused rat kidney (46). In contrast, in isolated rabbit afferent arterioles, extracellular calcium has been shown to be less important in the contractile response to NE than to ANG II (28).

Other mechanisms may underlie the differing inhibitory effects of nifedipine and the calcium-free medium. For example, our results do not exclude the possibility that the short-term, calcium-free medium interacts with calcium release from internal stores. There is little doubt that prolonged EGTA treatment impacts on the filling of calcium stores secondary to attenuated entry. Such an inhibitory effect is consistent with reported positive interactions such as calcium release-induced calcium entry and calcium entry stimulation of calcium release (41, 53) (S. K. Fellner and W. J. Arendshorst, unpublished observations). Many published studies indicate that nifedipine can rapidly and completely block calcium entry via L-type channels and that EGTA effectively chelates extracellular calcium with no major immediate effect on mobilization of calcium from intracellular stores. Studies conducted in our laboratory and those of others indicate that the employed doses of nifedipine and EGTA are maximal in that they completely inhibit calcium entry via L-type channels and contraction stimulated, for example, by ANG II (5, 26, 27, 32, 48). Also, both of these interventions are known to effectively abolish calcium entry triggered by K$^{+}$-induced depolarization in afferent arteriolar vascular smooth muscle cells (Fig. 5) (6, 26, 31, 48). In contrast, a low-Ca$^{2+}$ medium does not affect contraction and calcium signaling in the efferent arteriole, a vessel thought to depend primarily on intracellular release (10, 11). Several laboratories have provided evidence for selective action of extracellular calcium on entry pathways, in that reductions in extracellular calcium using short-term exposure to EGTA are shown to have no major effect on IP$_3$-induced and thapsigargin-sensitive mobilization of calcium from intracellular stores (10, 20, 53).
With regard to the relative importance of calcium mobilization from intracellular stores, we found that TMB-8, an inhibitor of IP$_3$-mediated calcium release from intracellular stores, attenuated the renal vasoconstriction produced by NE in a dose-dependent manner. The two highest doses of TMB-8 had similar blocking effects, suggesting near maximal inhibition. These findings provide evidence that 70–80% of the NE-induced increase in renal vascular resistance depends on mobilization of [Ca$^{2+}$], from internal stores. As a comparison with other vasoconstrictor agents, earlier blood flow studies conducted in our Chapel Hill laboratory showed that TMB-8 maximally antagonized ~50% of the renal vasoconstriction produced by ANG II or by vasopressin (15, 43). Therefore, it is reasonable to conclude that calcium mobilization from intracellular stores plays a more important role for the renal vascular response elicited by NE than by ANG II and vasopressin.

Consistent with our in vivo studies, calcium mobilization was important to NE-induced increases in [Ca$^{2+}$] in our isolated rat afferent arterioles. After a 2-min pretreatment with TMB-8, the arteriolar [Ca$^{2+}$] response to NE was blunted at 10–15 s; the sustained phase at 30–35 s was abolished. In other studies, we tested the specificity of TMB-8 to antagonize the [Ca$^{2+}$] increase at 30–35 s, which had been blocked by thapsigargin, whereas the contractile response elicited by depolarization with 50 mM KCl in the medium. Our results clearly show that TMB-8 had no demonstrable effect on the calcium response and, therefore, on calcium entry through voltage-sensitive calcium entry channels. The lack of effect was noted whether the drug was introduced either before or after stimulation with 50 mM KCl. Supportive evidence derives from blood flow studies in which TMB-8, administered at doses blocking vasopressin-induced renal vasoconstriction, had no effect on vasorelaxation caused by (+)-BAY K8644 and its stimulation of L-type calcium entry channels (15). A strong dependence of NE effects on calcium mobilization is demonstrated by a larger inhibition of NE- than ANG II-induced contraction of juxtaglomerular resistance vessels of juxtamedullary nephrons by thapsigargin, an agent that depletes intracellular stores by blocking calcium uptake into the sarcoplasmic reticulum (23). The response to NE was almost totally blocked by thapsigargin, whereas the contractile response to ANG II was attenuated to 50% by the same treatment. Our blood flow observations for the rat are in general agreement with previous studies examining the role of calcium mobilization from internal stores in other species. In anesthetized dogs, TMB-8 effectively inhibited the renal vasoconstriction produced by NE, but did not interfere with blood flow autoregulation (40). In this study, TMB-8 produced a dose-dependent inhibition of the NE-elicited vasoconstriction of 50% and 80%. Another study in dogs reported that TMB-8 affects sodium excretion and blocks some of the antinatriuretic effect of renal nerve stimulation in the dog (38). In the absence of hemodynamic data and effects on filtered load and tubular reabsorption, it is difficult to distinguish between vascular and tubular effects. Interestingly, NE is reported to stimulate IP$_3$ production in renal cortical slices, as well as vascular smooth muscle cells (19, 36).

Combined treatment with nifedipine and TMB-8 produced no greater inhibition of NE-induced reductions in blood flow than each agent did when administered alone. These results indicate that the two identifiable signaling mechanisms probably interacted, rather than being independent of each other. These results contrast with earlier hemodynamic studies, in which signal transduction pathways were evaluated in response to stimulation by ANG II and vasopressin (15, 43). The vascular response to NE appears to involve a more complex signaling pattern, in which there is continuous crosstalk between calcium mobilization and entry. Previous results have suggested complex signaling pathways in the renal vasculature (8). Based on studies of the isolated perfused kidney preparation, NE elicits calcium mobilization both from intra- and extracellular stores through activation of α1 receptors. Our in vivo and in vitro observations are internally consistent. An interaction is demonstrated by the ability of TMB-8 to abolish the sustained [Ca$^{2+}$] plateau triggered by NE in isolated afferent arterioles. The commonly accepted general scheme for vascular smooth muscle cells holds that calcium entry is solely responsible for the sustained phase of increases in [Ca$^{2+}$]; and that inhibition of calcium mobilization should not have a major impact on the period of sustained stimulation. Moreover, several reports in the literature suggest different calcium signaling pathways in large-diameter conduit vessels and in resistance vessels in the microcirculation (9, 37, 52). In renal microvessels, it has recently been proposed that chloride channels may play a role in the recruitment of [Ca$^{2+}$]; (28, 49). Further studies are needed to more completely elucidate the precise mechanisms, and their interactions, that lead to calcium recruitment in the regulation of the renal hemodynamic response to adrenergic receptor stimulation.

In summary, our study presents a unique combination of in vitro and in vivo studies that address calcium signaling mechanisms and stimulation by activation of adrenergic receptors in renal resistance vessels. There is general agreement between these two different preparations with respect to the action of NE and the blocking of its action with nifedipine and TMB-8. Both the NE-induced [Ca$^{2+}$]; response in isolated afferent arterioles and the renal vasoconstriction in vivo are attenuated by nifedipine to ~50%. These results suggest the importance of L-type voltage-gated calcium channels in the renal vascular response to activation of adrenoceptors. The existence of voltage-gated calcium channels is further confirmed by the fact that K$^+$-induced depolarization of isolated vessels causes calcium entry via a nifedipine-sensitive pathway. Exposure of the isolated vessel to a nominally calcium-free solution results in a higher degree of attenuation of the
NE-induced increase in [Ca^{2+}], suggesting alternative calcium entry pathways. TMB-8 blocks ~80% of the NE response, both in vivo and in vitro. In vivo experiments established that there is no additive effect of combined treatment with nifedipine and TMB-8, indicating a continuous and complex interplay between calcium mobilization and entry.

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