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 adrenoceptor activation. We measured renal blood flow (RBF) in Sprague-Dawley rats in vivo using electromagnetic flowmetry. We measured intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]) 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$^{2+}$ entry channels. 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$^{2+}$], 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$^{2+}$] response to NE. The increased [Ca$^{2+}$] elicited 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 adrenoceptor 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; adrenoceptor; renal circulation; afferent arteriole; 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester; nifedipine; vascular smooth muscle

The sympathetic autonomic system plays a crucial role in the control of renal hemodynamics, glomerular ultrafiltration, and the short- and long-term regulation of extracellular fluid volume and arterial blood pressure. In addition, the release of renin from the granular cells located in the media of the distal end of the afferent arteriole is controlled, in part, by stimulation of renal adrenoceptors (12, 16). The kidneys are highly endowed with sympathetic nerves that extend primarily to the renal vasculature (3). Catecholamines released from nerve terminals and of humoral origin exert their effect by activation of cell surface adrenoceptors on smooth muscle cells to produce changes in the intracellular free cytosolic calcium concentration ([Ca$^{2+}$]), resulting in a contractile response by renal resistance vessels. Binding of Ca$^{2+}$ to calmodulin, followed by a change in calmodulin structure, activates myosin light-chain kinase, which causes an increase in smooth muscle cell tone (50). The increase in [Ca$^{2+}$] induced by activation of cell surface receptors is thought to be mediated by the recruitment of Ca$^{2+}$ from one or two major sources, i.e., mobilization from intracellular stores and entry from the extracellular space through voltage-dependent and/or receptor-activated calcium channels located in the cell membrane. In smooth muscle cells, there are two forms of internal Ca$^{2+}$ stores: the sarcoplasmatic reticulum and the mitochondria (34). The latter is not believed to contribute significantly to the regulation of [Ca$^{2+}$] in smooth muscle cells. Two channels for the release of Ca$^{2+}$ from sarcoplasmic reticulum have been described: a ryanodine receptor and an inositol 1,4,5-trisphosphate (IP$_3$) receptor. The IP$_3$ receptor is present in all smooth muscle cells, whereas ryanodine receptors do not seem to be present in large numbers in smooth muscle cells from all tissues.

The relative importance of entry vs. mobilization in different smooth muscle cell preparations, in response to stimulation with catecholamines, is controversial (9, 37, 52). Differences in results may be due to different methods of preparation of vessels and cells, or it may be that cells subpassaged in culture may undergo phenotypic changes. Thus information obtained through in vitro experiments may not be identical to mechanisms regulating the tone of resistance vessels in vivo. Reports from a variety of animals (i.e., rat, dog, cat, and monkey) show that an increase in renal sympathetic nerve activity decreases renal blood flow (RBF) and increases renal vascular resistance (12, 16). Renal vasoconstriction elicited by high-frequency nerve stimulation and norepinephrine (NE) administration is blocked by a calcium entry antagonist (21, 33, 39, 47).

Attempts to study the glomerular arterioles have long been hampered by technical problems, mostly related to the small size and inaccessibility of these vessels. However, during the last decade, successful adaptation of the isolated and perfused tubule technique has facilitated direct measurements of the glomerular arteriolar diameter (25, 28, 29, 51, 55) and of [Ca$^{2+}$] in the smooth muscle cells of these vessels (6, 11, 29, 45). Through the use of this technique, isolated afferent and efferent arterioles from rat and rabbit were found to constrict and exhibit an increase in smooth muscle cell [Ca$^{2+}$], in response to NE stimulation (13, 29, 45, 55). Recently, results obtained on the in...
vitro blood-perfused juxtamедullary 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 $[\text{Ca}^{2+}]_i$, and contraction in renal resistance vessels in response to stimulation of adrenoceptors. The pharmacological agents nifedipine and 3,4,5-trimethoxybenzoic acid-8-(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 $[\text{Ca}^{2+}]_i$ 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 isocnictic BSA (47 g/l) to replace losses associated with surgery (1.25 ml/100 g body wt). Thereafter, isocnictal albumin (10 µl/min) was infused continuously, for the duration of the experiment, to maintain hematoctrit 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 cm into the left renal artery. The catheter was connected to a noncannulating electromagnetic flow probe (Carolina Medical Electronics) and the renal arterial catheter to administer test agents directly to the kidney before gaining access to extrarenal sites. An animal was discarded if NE affected arterial pressure. Throughout the experiment, heparinized (30 U/ml) isotonic saline was infused (5 µl/min) via the renal arterial catheter. A noncannulating flow probe was 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 dissolved 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.

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. 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 dissection dish containing an ice-chilled physiological salt solution (PSS), with BSA (Sigma) added to give a final concentration of 2 µM and Pluronic F127 (0.01%; Molecular Probes) in PSS. After completion of the dissection procedure, a vessel was loaded with fura 2 acetomethoxy ester (fura 2-AM) for 45–60 min, in the dark, at room temperature, as previously described (24, 45). Fura 2-AM 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 perfused 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...
the back of the pipettes to generate negative pressure. For measurements of [Ca$^{2+}$], the arteriole was centered in the optical field of a ×40 quartz oil-immersion objective. The preparation was visualized by video camera (Sony) and monitor. Variable shutters were adjusted to center an arteriole in the sampling window. This arrangement made possible continuous control of the position of the preparation throughout an experiment. The arteriole 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 [Ca$^{2+}$], was calculated based on the ratio at 340/380 nm, according to the equation described by Grynkiewicz et al. (18): [Ca$^{2+}$] = Kd·[(R - R$_{min}$)/(R$_{max}$ - R)]·(Sf/Sb), where Kd is the dissociation constant of fura 2 for calcium; Sf and Sb are the 380-nm fluorescence at saturating and zero calcium concentrations, respectively; and R$_{min}$ and R$_{max}$ are values of R (fluorescence ratio 340/380) at low and at saturating calcium concentration, respectively. Values for Kd, R$_{min}$, R$_{max}$, Sf, and Sb 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 solution in the renal vascular preparation. The arteriole was centered in the optical field of a 40× quartz oil-immersion objective. 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 adrenoeceptor 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 (A, n = 12), 3,4,5-trimethoxybenzoic acid-8-(diethylaminol)octyl ester (TMB-8; B, n = 8), and nifedipine + TMB-8 (C, n = 6) on renal hemodynamic response to injection of norepinephrine (NE) into renal artery. Values are means ± SE. At least 6 animals were used in each group. *P < 0.05 vs. NE alone. #P < 0.01 vs. lowest concentration of antagonist (A and B); vs. nifedipine (C, 750 ng).
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 IP3-induced calcium mobilization from the sarcoplasmic reticulum in the renal vascular response to activation of adrenoceptors by NE. Administration of NE during the control period produced a response to activation of adrenoceptors by NE. Administration of NE within the standard time interval (5 min) reduced the NE effect to 17 ± 3% of basal flow (P < 0.001 vs. NE). 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 \([\text{Ca}^{2+}]\), 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 adrenergic receptors, 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 \([\text{Ca}^{2+}]\) 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 \([\text{Ca}^{2+}]\) from 94 ± 14 to 217 ± 26 nM at 10–15 s (255%) and to 216 ± 27 nM at 30–35 s (244%; \(P < 0.001\)). After short-term exposure to the calcium-free solution, baseline levels of \([\text{Ca}^{2+}]\) decreased from 100 ± 17 to 85 ± 15 nM (\(P < 0.05\)). NE challenge in the presence of the calcium-free solution for 50 s caused a small but significant (40%) increase in \([\text{Ca}^{2+}]\), at 10–15 s only (from 85 ± 15 to 110 ± 18 nM; \(P < 0.01, n = 7\)). After continued NE stimulation for 30–35 s, the change in \([\text{Ca}^{2+}]\) decayed somewhat, such that the late plateau phase did not differ from control \([\text{Ca}^{2+}]\), (98 ± 18 vs. 85 ± 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 \([\text{Ca}^{2+}]\), from 141 ± 24 to 251 ± 32 nM and 246 ± 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 \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\) was close to 35%. The absolute values increased from 132 ± 17 to 176 ± 24 nM and to 175 ± 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 \([\text{Ca}^{2+}]\), from 101 ± 15 to 216 ± 31 nM at 10–15 s and to 217 ± 30 nM at 30–35 s (\(P < 0.001\)). Two minutes of exposure to TMB-8 did not affect baseline \([\text{Ca}^{2+}]\), but did significantly attenuate the \([\text{Ca}^{2+}]\) response to NE, both at 10–15 s and 30–35 s (\(P < 0.05\)). As Fig. 3B shows, \([\text{Ca}^{2+}]\), rose from 103 ± 18 to 130 ± 27 nM at 10–15 s and to 124 ± 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 \([\text{Ca}^{2+}]\), during continued adrenergic receptor stimulation. These experiments enabled us to independently compare the effects of these agents on the initial rise and the sustained increase in \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), plateau was completely depen-
sudden decrease in 

Antagonism of L-type calcium channels produced a

The group means are shown in Fig. 4. In the absence of

NE containing PSS was replaced with the nominally
calcium-free solution containing NE, the 

immediately decreased to 74 ± 9 nM, a value roughly 10%
below control (n = 4).

As the individual experiment in Fig. 2B shows, the
response to NE was attenuated by subsequent administra-
tion of nifedipine. The group averages shown in Fig.
4 indicate that NE caused a rise in [Ca²⁺], 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 [Ca²⁺], 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
[Ca²⁺] is dependent on dihydropyridine-sensitive cal-
dium 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 [Ca²⁺] plateau.
The group means are shown in Fig. 4. In the absence of
the inhibitor, NE elicited a 90% rise in [Ca²⁺], from
89 ± 7 to 163 ± 21 nM (n = 5). On the addition of
TMB-8 + NE, the [Ca²⁺] 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
such channels, we performed experiments in which
smooth muscle cell membranes were depolarized using
a solution high in potassium (K50). The results estab-
lish that K50 initiated an 85% increase in [Ca²⁺], 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 [Ca²⁺], 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 [Ca²⁺] response to the depolarizing
treatment. The basal [Ca²⁺], 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 experi-
ments. The K50-induced elevation in [Ca²⁺], from 103 ±
27 to 156 ± 28 nM (164 ± 35%; n = 5, P < 0.03) was
totally abolished by the addition of nifedipine as [Ca²⁺],
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 per-
formed additional experiments to rule out the possibil-
ity that the blocking effect of this substance, both in

Fig. 5. Stimulatory effect of 50 mM KCl (K 50) on cytosolic calcium
concentration in rat afferent arterioles. A: nifedipine (50 s) abolished
effect of high KCl. B: TMB-8 was without effect on either immediate
(10–15 s) or sustained (30–50 s) calcium responses to high KCl.
Control values are percent change of baseline cytosolic calcium
concentration before 50 mM KCl, with or without pretreatment. *P <
0.05 vs. baseline; #P < 0.05 vs. 50 mM KCl without treatment. Values
are means ± SE for at least 5 experiments in each group.
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 that mediate 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 administration 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 constrictor 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 hydrenphrotic rat kidney, vasoconstriction elicited by NE and ANG II is almost completely reversed by the administration of 10$^{-6}$ M of three different dihydropyridines (21). During maximal vasodilation in the basal 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
The temporal response reported by these investigators tightly correlated with changes in vessel diameter (54). Juxtamedullary nephrons of the rat kidney, NE-induced and 3). Increases in \([\text{Ca}^{2+}]\) poststimulation were maintained at 30–35 s (Figs. 2 and 3). Increases in \([\text{Ca}^{2+}]\) in vascular smooth muscle cells are normally associated with muscle contractile force and constriction (4). Using confocal microscopy to estimate changes in \([\text{Ca}^{2+}]\) in pregglomerular vessels in juxtamедullary nephrons of the rat kidney, NE-induced changes in afferent arteriolar \([\text{Ca}^{2+}]\), were found to be tightly correlated with changes in vessel diameter (54). The temporal response reported by these investigators was a rapid increase in \([\text{Ca}^{2+}]\), followed by oscillating waves of elevated \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\) 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 attenuated the \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\) during stimulation with a high-potassium medium. This view is reinforced by the finding that nifedipine completely blocks the \([\text{Ca}^{2+}]\) response to depolarization induced by a high-potassium solution. This observation also excludes possible nonspecific 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 \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), 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 \([\text{Ca}^{2+}]\), 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 concentration 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). 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 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-\([\text{Ca}^{2+}]\) 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 IP3-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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ in our isolated rat afferent arterioles. After a 2-min pretreatment with TMB-8, the arteriolar $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ release, as several investigators have suggested additional effects (1, 24, 42). For this purpose, we evaluated whether the dose of TMB-8 employed in our studies had any effect on calcium entry stimulated 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 vasoconstriction 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 preglomerular 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-elicted 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 IP3 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 $\alpha_1$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ (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 $[\text{Ca}^{2+}]_i$ 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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