L-type calcium channels in the renal microcirculatory response to endothelin

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The renal circulation contains endothelin (ET) type A and B (ET<sub>A</sub> and ET<sub>B</sub>) receptors, which can mediate vasoconstriction. Although it is generally accepted that ET<sub>A</sub> receptor activation stimulates mobilization of Ca<sup>2+</sup><sup>+</sup> from extracellular and intracellular sources (28), there is very little information about ET<sub>B</sub> receptor subtype signaling pathways in vascular smooth muscle. Both receptor subtypes stimulate G protein-dependent pathways. Although a number of mediators have been identified for their involvement in the Ca<sup>2+</sup><sup>+</sup> mobilization response to ET-1, the precise function and coordination of these messengers is not fully resolved. In the vasculature, the assumption that the ET<sub>A</sub> receptor is the predominant subtype underlies a number of studies investigating the nature of the vasoconstrictor actions of ET-1 (28). However, ET<sub>A</sub> and ET<sub>B</sub> receptors contribute to the renal vasoconstrictor actions of ET-1, and the extent to which the signal transduction mechanisms may be receptor subtype specific is unknown.

Cao and Banks (1) reported that Ca<sup>2+</sup><sup>+</sup> channel antagonists prevent the systemic hemodynamic actions of ET-1 but have no effect on ET-1-induced renal vasoconstriction. Their results are consistent with the hypothesis that ET<sub>B</sub> receptor-mediated vasoconstriction does not depend on extracellular Ca<sup>2+</sup><sup>+</sup>, because they used high doses of ET-1, which primarily activate ET<sub>B</sub> receptors in the kidney. On the other hand, Loutzenhiser et al. (22) reported that Ca<sup>2+</sup><sup>+</sup> channel blockade completely reversed ET-1-induced vasoconstriction in the isolated perfused hydromephonetic kidney. In isolated rabbit arterioles, Edwards et al. (5) observed that ET-1-induced contraction of afferent, but not efferent, arterioles was sensitive to Ca<sup>2+</sup><sup>+</sup> channel antagonists. In isolated smooth muscle cells obtained from microdissected interlobar and arcuate arteries of the rat, Gordienko et al. (10) observed that ET-1 increased cytosolic Ca<sup>2+</sup><sup>+</sup> via intracellular and extracellular sources. They also provided evidence that ET-1 activates T- and L-type channels as a means of stimulating extracellular Ca<sup>2+</sup><sup>+</sup> influx.

Touyz et al. (35) reported that ET<sub>B</sub> receptor-mediated contraction in small mesenteric arteries is mediated by increases in intracellular Ca<sup>2+</sup><sup>+</sup>. However, ET<sub>B</sub> receptor signaling systems may not be ubiquitous among cell types. As an example, Wu-Wong et al. (36, 38) reported that ET<sub>B</sub> receptors do not stimulate Ca<sup>2+</sup><sup>+</sup> mobilization in astrocytoma cells. Although a large number of ET<sub>B</sub> receptors are present in the kidney, primarily in tubular epithelium, there is only limited information regarding the Ca<sup>2+</sup><sup>+</sup> signaling pathways during ET<sub>B</sub> receptor-mediated renal vasoconstriction. Our laboratory has reported that, in contrast to ET-1, ET-3, the natural ligand for the ET<sub>B</sub> receptor, has no effect on intracellular Ca<sup>2+</sup><sup>+</sup> in isolated pregglomerular vascular smooth muscle cells (32). In contrast, using a similar preparation, Fellner and Arendshorst (8) recently reported that the ET<sub>B</sub> receptor agonist IRL-1620 increases cytosolic Ca<sup>2+</sup><sup>+</sup> in a fashion nearly identical to ET-1.

The purpose of the present study was to determine the role of L-type Ca<sup>2+</sup><sup>+</sup> channels in the renal hemodynamic response to ET-1 and specific ET<sub>B</sub> receptor activation by in vivo and in vitro approaches. The effect of Ca<sup>2+</sup><sup>+</sup> channel blockade on renal vasoconstrictor responses to direct intra-arterial administration of ET-1 and the ET<sub>B</sub> receptor agonist sarafotoxin 6c (S6c) were determined in anesthetized rats. In addition, the role of L-type Ca<sup>2+</sup><sup>+</sup> channels in response to ET-1 was determined in the isolated blood-perfused juxtaglomerular nephron preparation and in isolated pregglomerular microvascular smooth muscle cells.

METHODS

The Institutional Animal Care and Use Committee at the Medical College of Georgia approved these studies.

In vivo hemodynamic experiments. Male Sprague-Dawley rats (225–250 g body wt; Harlan Laboratories, Indianapolis, IN) were anesthetized with thiobutabarbital (Inactin, 50 mg/kg) and placed on
a servo-controlled heating table to maintain a constant temperature of 37°C. A tracheotomy was performed to facilitate unobstructed breathing. The left jugular vein was cannulated for infusion of BSA (6.2%) in saline (0.9% NaCl) and the ET<sub>1</sub> receptor antagonist A-192621, while the right femoral artery was cannulated to monitor mean arterial pressure (MAP) with a MacLab data acquisition system. After cannulation of the right femoral artery, a microcatheter was advanced ~3 mm into the left renal artery for intrarenal infusion of nifedipine, ET-1, or S6c. An ultrasonic flow probe (Transonic Systems) was placed on the left renal artery to measure renal blood flow (RBF). After a 60-min equilibration period, rats were given a bolus of 0.9% NaCl or A-192621 (30 mg/kg) via the jugular vein. Three 30-min periods followed in which ET-1 or S6c (1, 10, and 100 pmol) were given intrarterially in the presence or absence of nifedipine (1.5 μg). This dose and method of nifedipine administration have been previously shown to block 90% of the maximum decrease in RBF produced by the voltage-gated Ca<sup>2+</sup> channel activator BAY K 8644 (30).

In vitro blood-perfused juxtamedullary nephron experiments. Experiments were conducted, in vitro, using the blood-perfused juxtamedullary nephron technique, as previously described (3, 13, 14). Two male Sprague-Dawley rats (350–400 g body wt) were used for each experiment. Rats were anesthetized with pentobarbital sodium (40 mg/kg ip), and perfusate blood was collected and prepared as previously described (13, 14). Briefly, blood was collected from the nephrectomized blood donor rat into a heparinized (500 U) syringe. The plasma and erythrocyte fractions were separated, and the leukocyte fraction was discarded. Washed erythrocytes were combined with the filtered (0.2-μm exclusion) plasma to yield a hematocrit of ~33%. The reconstituted blood was filtered through a 5-μm nylon mesh and saved for later use.

The right renal artery of the kidney donor was cannulated and perfused with a Tyrode buffer solution containing 5.2% BSA and a complement of L-amino acids (18). The perfused kidney was removed and sectioned along the longitudinal axis, with care taken to leave the papilla intact on the dorsal two-thirds of the kidney (3). The papilla was reflected and the pelvic mucosa was removed to expose the main cortical and papillary tissue.

Intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) was measured in single pregemlular smooth muscle cells. Experiments were performed using standard microscope-based fluorescence spectrophotometry techniques as previously described (15, 16). The excitation wavelengths were set at 340 and 380 nm, and the emitted light was collected at 510 ± 20 nm. Fluorescence intensity was collected (5 data points/s) and analyzed with the aid of Photon Technology International software. Fluorescence data were calibrated in vitro according to the method used by Grynkiewicz et al. (12).

Intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in single preglomerular smooth muscle cells was measured as described previously (15, 16, 19). Suspensions of microvascular smooth muscle cells were loaded with 10 μM fura 2-AM (Molecular Probes, Eugene, OR), and an aliquot of cell suspension was transferred to the perfusion chamber (Warner Instrument, Hamden, CT) and mounted on the stage of a Nikon Diaphot inverted microscope. The cells were superfused at 37°C with a control physiological salt solution of the following composition: 125 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM glucose, 20 mM HEPES, 1.8 mM CaCl<sub>2</sub>, and 0.1% BSA. For each experiment, fluorescence data were collected from a single cell after background subtraction. A new coverslip was used for each experiment.

The effect of ET on [Ca<sup>2+</sup>]<sub>i</sub> was determined by exposure of single cells to normal-Ca<sup>2+</sup> physiological salt solutions containing 100 mM ET-1, 1 μM S6c, or another ET<sub>1</sub> receptor agonist, IRL-1620, at 10 μM. ET-1 was tested in the presence and absence of 10 μM diltiazem. Diltiazem, instead of nifedipine, was used to avoid nifedipine-related autofluorescence at the excitation wavelengths for fura 2. Previous studies establish that 10 μM diltiazem effectively inhibits the Ca<sup>2+</sup> influx response evoked by exposure of pregemlular smooth muscle cells to 90 mM K<sup>Cl</sup> (16).

Cells were also superfused with a nominally Ca<sup>2+</sup>-free solution (Ca<sup>2+</sup>-free physiological salt solution), which resembled the physiological salt solutions, except no CaCl<sub>2</sub> was added. No EGTA was added to the solution. Previous studies have shown that exposure of pregemlular microvascular smooth muscle cells to 90 mM K<sup>Cl</sup> in a similarly Ca<sup>2+</sup>-free solution resulted in no detectable increase in [Ca<sup>2+</sup>]<sub>i</sub> (16).

S6c, IRL-1620, and ET-1 were obtained from American Peptide, collagenase from Boehringer Ingelheim Chemicals (Petersburg, VA), and BSA from Calbiochem (La Jolla, CA). All other reagents were obtained from Sigma (St. Louis, MO). Values are means ± SE or representative traces. Within-group comparisons were assessed by
ANOVA for repeated measures; differences between groups were analyzed with Fisher’s protected least square difference post hoc tests. Significant differences were noted when $P < 0.05$.

RESULTS

In vivo hemodynamic experiments. Intrarenal injection of ET-1 produced dose-dependent decreases in RBF (Fig. 1A). Coadminstration of nifedipine had no effect on the response to ET-1 at the two lower doses; yet the decrease in RBF produced by ET-1 was significantly attenuated at the highest dose by $\sim 50\%$. Intrarenal injection of S6c also produced a dose-dependent decrease in RBF (Fig. 1B). In contrast to ET-1, the highest dose of S6c produced a biphasic vasoconstriction typified by a rapid, pronounced vasoconstriction followed by a partial recovery to an intermediate plateau level. Previous studies demonstrated a net vasoconstrictor effect of ETB receptor agonists, despite evidence for vasodilator and vasoconstrictor actions within the renal circulation (26). Coadministration with the $\Ca^{2+}$ channel blocker nifedipine had no significant effect on the renal vasoconstrictor response to the lower doses of S6c. However, nifedipine significantly attenuated the decrease in RBF produced by the highest dose of S6c. To verify that S6c was producing the vasoconstriction through the ETB receptor, A-192621 was administered 15 min before injection of S6c in a separate group of rats (Fig. 1C). A-192621 alone produced a slowly developing decrease in RBF consistent with a tonic influence of ETB receptors to maintain an endothelium-dependent vasodilatory influence within the renal circulation and increased ETA receptor activation associated with reduced clearance of endogenous ET-1. S6c had no effect on RBF after A-192621 treatment.

Intrarenal ET-1 infusion had no significant effect on MAP (Fig. 2A). MAP did not significantly change after intrarenal infusion of S6c at the two lower doses (Fig. 2B). However, the higher dose produced a small, transient decrease in MAP followed by a more prolonged elevation in blood pressure. The hypotensive response was exaggerated in rats given nifedipine, although the increase in arterial pressure was unaffected. A-192621 increased MAP and prevented any change in MAP after S6c injection at all doses (Fig. 2C).

In vitro blood-perfused juxtamedullary nephron experiments. The effect of $\Ca^{2+}$ channel blockade on the afferent arteriolar response to ET-1 was directly assessed, in vitro, using the blood-perfused juxtamedullary nephron technique. Two groups of arterioles were studied. The baseline diameters of the control and diltiazem-treated arterioles were similar and averaged $16.8 \pm 0.7$ and $18.8 \pm 1.1 \mu m$, respectively. Diltiazem increased afferent diameter significantly by $\sim 42\%$ to $26.5 \pm 1.3 \mu m$. Exposure of afferent arterioles to increasing concentrations of ET-1 (1 pM–10 nM) produced concentration-dependent vasoconstriction under control conditions (Fig. 3). Diameter decreased by $12 \pm 2$, $25 \pm 3$, $40 \pm 4$, $65 \pm 4$, and $78 \pm 2\%$, respectively, with each successive increase in ET-1 concentration. Diltiazem treatment significantly attenuated the vasoconstrictor response evoked by ET-1 at 1 and 10 pM but had no significant effect on vasoconstrictor responses to higher concentrations.

Isolated microvascular smooth muscle cell experiments. The effect of $\Ca^{2+}$ channel blockade on $\Ca^{2+}$ signaling responses elicited by ET-1 was directly assessed using freshly isolated pregemular smooth muscle cells. A total of 84 individual
cells prepared from 18 tissue dispersions were examined. Baseline $[\text{Ca}^{2+}]_i$ averaged $104 \pm 3 \text{nM}$, which is similar to values reported previously (15, 16, 19). S6c at 1 $\mu$M had no significant effect on $[\text{Ca}^{2+}]_i$ (Fig. 4A), but ET-1 treatment at 100 nM in the same cell resulted in a marked increase in $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ increased an average of $34 \pm 19 \text{nM}$ during exposure to 1 $\mu$M S6c ($n = 9$ cells from 4 dispersions). In separate experiments, we examined the effect of IRL-1620 on $[\text{Ca}^{2+}]_i$ in preglomerular smooth muscle cells. IRL-1620 at 10 $\mu$M evoked a minimal increase in $[\text{Ca}^{2+}]_i$, whereas 100 nM ET-1 produced a robust and significantly greater $\text{Ca}^{2+}$ response (Fig. 4B). Peak increases in $[\text{Ca}^{2+}]_i$, induced by IRL-1620 averaged $26 \pm 5 \text{nM}$ ($n = 8$) under control conditions and $39 \pm 4 \text{nM}$ ($n = 25$) in the presence of diltiazem.

Fig. 4. Typical intracellular $\text{Ca}^{2+}$ recordings in response to S6c (A) or IRL-1620 (B) in single preglomerular vascular smooth muscle cells. Horizontal bars indicate administration of S6c and IRL-1620.
Similarly prepared cells responded to ET-1 with a marked increase in $[\text{Ca}^{2+}]_i$. ET-1 at 100 nM increased $[\text{Ca}^{2+}]_i$ from a baseline of 120 nM to a peak value of 895 nM before stabilization at a sustained level of 127 nM (Fig. 5A). Group analysis revealed that ET-1 increased $[\text{Ca}^{2+}]_i$, from a baseline of 94 ± 5 nM to a maximum of 577 ± 70 nM ($P < 0.001$ vs. baseline, $n = 23$ cells from 9 dispersions). This response profile was unaltered by pretreatment with the $\text{Ca}^{2+}$ channel blocker diltiazem ($10 \mu\text{M}$). Consistent with responses from untreated control cells and with the example trace shown in Fig. 5B, 100 nM ET-1 increased $[\text{Ca}^{2+}]_i$ from a baseline of 104 ± 4 to a peak of 592 ± 95 nM. Similar results were obtained when more general blockade of $\text{Ca}^{2+}$ channels was imposed by the introduction of 5 mM Ni$^{2+}$ to the bathing medium. These responses, along with the effects of diltiazem, are summarized in Fig. 6. Neither diltiazem nor Ni$^{2+}$ had an effect on the peak or sustained change in $[\text{Ca}^{2+}]_i$ evoked by ET-1 or on baseline $\text{Ca}^{2+}$ levels. However, 5 mM Ni$^{2+}$ completely blocked the $\text{Ca}^{2+}$ response to KCl. Under control conditions, 90 mM KCl increased $\text{Ca}^{2+}$ from 109 ± 12 to 203 ± 42 nM ($P < 0.05$), whereas $[\text{Ca}^{2+}]_i$, averaged 100 ± 15, 99 ± 14, and 94 ± 12 nM during the control, NiCl, and NiCl + KCl periods, respectively. We previously established the ability of 10 $\mu$M diltiazem to block the $\text{Ca}^{2+}$ response to 90 mM KCl (15, 16).

**DISCUSSION**

The present study utilized three model systems to determine the role of L-type $\text{Ca}^{2+}$ channels in the renal microvascular response to ET-1 and ET$_B$ receptor activation. In whole animal experiments, $\text{Ca}^{2+}$ channel blockade inhibited the vasoconstriction responses to the nonselective ligand ET-1 and the ET$_B$ receptor agonist S6c only at the highest dose. These findings are consistent with a relatively minor role for L-type $\text{Ca}^{2+}$ channels in the response to ET receptor activation by ET$_A$ or ET$_B$ receptors. In vitro responses of blood-perfused juxtamedullary afferent arterioles were slightly attenuated by $\text{Ca}^{2+}$ channel blockade only at very low concentrations of ET-1. These results are in general agreement with findings in isolated renal vascular smooth muscle cells that neither diltiazem nor Ni$^{2+}$ had an effect on the changes in $[\text{Ca}^{2+}]_i$ produced by ET-1. Selective activation of ET$_B$ receptors with S6c had no effect on $[\text{Ca}^{2+}]_i$. Collectively, these results clearly indicate that ET-1 and ET$_B$ receptor-dependent renal vasoconstriction only has a minor dependence on L-type $\text{Ca}^{2+}$ channels in the renal circulation. It is not clear why the minor influence of $\text{Ca}^{2+}$ channel blockade was observed only at the low concentrations in the juxtamedullary nephron preparation but only at high doses in the whole kidney preparation. The small differences in the contribution of L-type $\text{Ca}^{2+}$ channels in the different model systems may be related to contributions of other cell types or mediators that could modulate the response to ET-1 in the different preparations.

ET$_A$ receptor activation results in a biphasic increase in $[\text{Ca}^{2+}]_i$. In the first phase, ET-1 binds to a specific receptor at the cell surface, activating a pertussis toxin-insensitive G protein, which in turn stimulates phospholipase C. Hydrolysis of phosphatidylinositol by phospholipase C generates inositol triphosphate (IP$_3$) and diacylglycerol, both of which are known to function as second messengers, transducing information from the surface of the cell to the interior (28). IP$_3$ is a fast-acting mediator that facilitates the first phase of the response by binding to a receptor on the sarcoplasmic reticulum and triggering release of $\text{Ca}^{2+}$ from intracellular stores. In conjunction with the release of $\text{Ca}^{2+}$ from intracellular stores, a second phase is initiated, in which $\text{Ca}^{2+}$ moves into the cell from the extracellular space. The sustained elevation in intracellular $\text{Ca}^{2+}$ that results from $\text{Ca}^{2+}$ influx is thought to be responsible for increases in contractility. Although a number of
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Studies have attributed the activation of voltage-dependent, L-type Ca\textsuperscript{2+} channels to the preglomerular vasoconstriction evoked by numerous hormones, agonists, and autacoids (2, 7, 10, 16, 17, 19, 23, 24, 34), the precise mechanism by which ET activates this channel is largely unresolved. Furthermore, it has been shown that L-type Ca\textsuperscript{2+} channels account for only a portion of the Ca\textsuperscript{2+} response and that other channels may be involved (20, 33). In contrast to the IP\textsubscript{3}-mediated pathway by which intracellular Ca\textsuperscript{2+} is released, the purported processes by which extracellular Ca\textsuperscript{2+} enters vascular smooth muscle cells are less clearly defined.

The in vivo approach we used to study L-type Ca\textsuperscript{2+} channel involvement in renal vasoconstrictor responses to ET-1 has been effectively utilized to demonstrate the dependence of angiotensin II and other vasoconstrictors on L-type Ca\textsuperscript{2+} channels (9, 30, 31). These investigators were able to observe a significant inhibition of vasoconstrictor responses to angiotensin II, norepinephrine, and vasopressin when the Ca\textsuperscript{2+} channel blocker was injected along with the active peptide. In contrast to angiotensin II, ET-1 does not appear to be as dependent on L-type Ca\textsuperscript{2+} channels to produce renal vasoconstriction. This is somewhat surprising, because a number of reports indicate that the vasoconstrictor actions of angiotensin II can be inhibited by ET\textsubscript{A} receptor blockade (4, 29). However, these findings are consistent with reports that Ca\textsuperscript{2+} entry from extracellular sources may involve mechanisms other than L-type Ca\textsuperscript{2+} channels (20, 33). We previously reported that the sustained phase of ET-1-induced increases in intracellular Ca\textsuperscript{2+} is eliminated in a Ca\textsuperscript{2+}-free medium, indicating the requirement for extracellular Ca\textsuperscript{2+} (32). Because the present study demonstrates that this does not involve L-type Ca\textsuperscript{2+} channels, these data support an alternative mechanism for Ca\textsuperscript{2+} entry. These mechanisms appear to be unique to the renal vasculature, because dihydropyridine-sensitive Ca\textsuperscript{2+} channels are activated by ET-1 in other vascular beds (11, 21).

We previously reported that ET-3, a selective ET\textsubscript{B} receptor agonist at low doses, had no effect on intracellular Ca\textsuperscript{2+} levels in preglomerular smooth muscle cells, whereas ET-1, a non-selective ligand, produced a transient peak increase in intracellular Ca\textsuperscript{2+} consistent with IP\textsubscript{3}-mediated release of Ca\textsuperscript{2+} from intracellular stores (32). This was followed by slightly sustained increase that was more prolonged. Many vasoconstrictors can sustain this increase in intracellular Ca\textsuperscript{2+} by activating L-type Ca\textsuperscript{2+} channels to allow entry of extracellular Ca\textsuperscript{2+} (2, 7, 10, 16, 17, 19, 23, 24, 34). We also previously reported that the sustained, but not the peak, increase in intracellular Ca\textsuperscript{2+} is eliminated in the absence of extracellular Ca\textsuperscript{2+} (32). In the present study, we report that blockade of L-type Ca\textsuperscript{2+} channels had no effect on the peak or sustained rise in intracellular Ca\textsuperscript{2+}, suggesting an alternate pathway for Ca\textsuperscript{2+} entry in preglomerular vascular smooth muscle in response to ET-1.

Similar to the endogenous ligand ET-3, we observed that S6c, another selective ET\textsubscript{B} receptor agonist, had no effect on intracellular Ca\textsuperscript{2+}. These data indicate that ET\textsubscript{B} receptor-mediated constriction is through a Ca\textsuperscript{2+}-independent mechanism or occurs at sites other than interlobular arteries and afferent arterioles from which these cells are isolated. Endlich et al. (6) reported in the hydronephrotic kidney that ET\textsubscript{B} receptor-mediated vasoconstriction occurs primarily in efferent arterioles or sites in the afferent arteriole immediately adjacent to the glomerulus. Our observations that ET-3, IRL-1620, and S6c have no marked effect on intracellular Ca\textsuperscript{2+} contrast with a recent report of Fellner and Arendshorst (8) using the ET\textsubscript{B} receptor agonist IRL-1620 in a similar isolated cell preparation. In their report, IRL-1620 was the only ET\textsubscript{B} receptor agonist examined, and it produced a peak increase in intracellular Ca\textsuperscript{2+} of ~106 nM compared with the small but significant 26 nM change we observed. In contrast, our response to ET-1 was about twofold larger. The difference in results is not clear, but differences between IRL-1620 and other ET\textsubscript{B} receptor ligands have been reported in terms of binding characteristics to subpopulations of ET\textsubscript{B} receptors (25, 37). Interestingly, we previously reported considerably fewer IRL-1620 than ET-3 binding sites in the kidney (27).

In summary, using three different experimental approaches, our studies indicate a relatively minor role for L-type Ca\textsuperscript{2+} channel activation in the renal vasoconstrictor response to ET-1 and the selective ET\textsubscript{B} receptor agonist S6c. These data indicate that mechanisms unrelated to L-type Ca\textsuperscript{2+} channels contribute to ET-1-mediated vasoconstriction.

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REFERENCES