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Endothelin-A and -B receptors, superoxide, and Ca^{2+} signaling in afferent arterioles

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Fellner Susan K., and William Arendshorst. Endothelin-A and -B receptors, superoxide, and Ca^{2+} signaling in afferent arterioles. *Am J Physiol Renal Physiol* 292: F175–F184, 2007. First published June 20, 2006; doi:10.1152/ajprenal.00050.2006.—It is unknown if endothelin-A and -B receptors (ET_AR and ET_BR) activate the production of superoxide via NAD(P)H oxidase and subsequently stimulate the formation of cyclic adenine diphosphate ribose (cADPR) in afferent arterioles. Vessels were isolated from rat kidney and loaded with fura 2. Endothelin-1 (ET-1) rapidly increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by 303 nM. The superoxide dismutase mimetic tempol, the NAD(P)H oxidase inhibitor apocynin, and nicotinamide, an inhibitor of ADPR cyclase, diminished the response by ~60%. The ET_BR agonist sarafotoxin 6c (S6c) increased peak $[Ca^{2+}]_i$ by 117 nM. Subsequent addition of ET-1 in the continued presence of S6c caused an additional $[Ca^{2+}]_i$ peak of 225 nM. Neither nicotinamide or 8-bromo- (8-Br) cADPR nor apocynin decreased the $[Ca^{2+}]_i$ response to S6c, but inhibited the subsequent $[Ca^{2+}]_i$ response to ET-1. The ET_BR blockers BQ-788 and A-192621 prevented the S6c $[Ca^{2+}]_i$ peak and reduced the ET-1 response by more than one-half, suggesting an ET_BR/ET_AR interaction. In contrast, the ET_AR blocker BQ-123 had no effect on the S6c $[Ca^{2+}]_i$ peak and obliterated the subsequent ET-1 response. ET-1 immediately stimulated superoxide formation (measured with TEMPO-9-AC, 68 arbitrary units) that was inhibited 95% by apocynin or diphenyl iodonium. S6c or IRL-1620 increased superoxide by 8% of that caused by subsequent ET-1 addition. We conclude that ET_AR activation of afferent arterioles increases the formation of superoxide that accounts for ~60% of subsequent Ca^{2+} signaling. ET_BR activation appears to result in only minor increases in superoxide production. Nicotinamide and 8-Br-cADPR results suggest that ET-1 (and primarily ET_AR) causes the activation of vascular smooth muscle cell-ADPR cyclase.

renal microcirculation; TEMPO-9-AC; ryanodine receptor; cyclic adenine diphosphate ribose; reduced nicotinamide adenine dinucleotide phosphate oxidase; heterodimerization

IT HAS BECOME WELL ESTABLISHED that vascular smooth muscle cells (VSMC) of preglomerular vessels contain both endothelin-A and -B receptors (ET_AR and ET_BR). We have previously demonstrated that stimulation of preglomerular VSMC and afferent arteriole segments with the ET_BR selective agonist IRL-1620 or with endothelin-1 (ET-1) causes a rapid increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (9). Radioligand binding experiments suggest that ET_AR and ET_BR are expressed in rat preglomerular vessels in nearly equal proportions; however, membranes from both endothelial cells and VSMC were present (4). Such was also the case in membrane studies of both rat and rabbit renal microvessels, which showed that ET_AR and ET_BR are present in a proportion of 40:60 (5). Studies of the expression of ET_BR in the neonatal rat kidney demonstrate that, by *day 28* after birth, the interlobar artery develops immunofluorescent staining for ET_BR (37). Interestingly, in this study, the main renal artery (as well as mesenteric and pulmonary arteries) was devoid of staining for ET_BR, which suggests that conduit vessels express only ET_AR (37).

In vivo studies of the rat renal circulation show that the ET_BR agonists sarafotoxin 6c (S6c) and IRL-1620 produce vasoconstriction (21). Studies in the split hydronephrotic kidney model utilizing the ET_AR antagonist BQ-123, the ET_BR antagonist BQ-788, and IRL-1620 demonstrate that both ET_AR and ET_BR are present in afferent arterioles and that the effect of ET_BR activation causes twice as much vasoconstriction in the efferent arteriole (2, 6). Renal arterial administration of S6c causes a maximum reduction in renal blood flow of 56%; in contrast, the injection of the selective ET_AR antagonist FR139317 has no effect on basal renal blood flow (25). In contrast, a recent hemodynamic study of intrarenal injections of ET-1 or S6c produce a dose-dependent decline in renal blood flow (27). This study also examined the effect of S6c or IRL-1620 (10^{-6} M) on $[Ca^{2+}]_i$ in freshly isolated, single, preglomerular VSMC (27). Although these investigators note a robust, albeit delayed, response to ET-1 (10^{-7} M), they did not observe a significant $[Ca^{2+}]_i$ effect of S6c or IRL-1620. Studies from the same laboratory, performed with the isolated juxtamedullary preparation in which agonists and antagonists are added to the bath solution, confirm that both ET_AR and ET_BR activation lead to afferent arteriolar constriction (19).

Although it is clear that both ET_AR and ET_BR are present on the VSMC of the renal microcirculation and that the distribution of receptors may differ between afferent and efferent arterioles, it is not known if the consequences of receptor activation of the two subtypes differ. There is a paucity of information about the Ca^{2+} signaling pathways of ET-1 receptor stimulation in afferent arteriolar VSMC. Recently, our laboratory has become interested in peptide agonist activation of G protein-coupled receptors with downstream release of Ca^{2+} from the sarcoplasmic reticulum (SR). We have provided evidence that angiotensin II (ANG II) rapidly activates NAD(P)H oxidase, resulting in the formation of superoxide in afferent arterioles (10). Superoxide then results in the stimulation of the VSMC adenine diphosphate ribose (ADPR) cyclase to form cyclic ADPR (cADPR) (40). cADPR sensitizes the ryanodine receptor (RyR) to Ca^{2+} (calcium-induced calcium release) to enhance Ca^{2+} release from the SR (8, 10). One of us (S. K. F.) has shown that ET-1 utilizes the NAD(P)H oxidase, superoxide, and ADPR cyclase pathway in VSMC of the anterior mesenteric artery of *Squalus acanthias* (12).

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To investigate possible differences between ET_AR- and ET_BR-generated superoxide production and the resultant effect on Ca^{2+} signaling in rat afferent arterioles, we have utilized the ET_BR agonists S6c and IRL-1620, the ET_BR antagonists BQ-788 and A-192621, the superoxide dismutase (SOD) mimetic tempol, the NAD(P)H oxidase inhibitors apocynin and diphenyl iodonium (DPI), and the ADPR cyclase inhibitor nicotinamide. As well, we measured superoxide production following stimulation of afferent arterioles with ET-1 or ET_B agonists. To substantiate that endothelial cells are not functionally active in this preparation of afferent arteriolar segments, we performed studies in the presence and absence of N^{ω}-nitro-L-arginine methyl ester (L-NAME) and measured the $[Ca^{2+}]_i$ responses to bradykinin (BK) (10^{-7}-10^{-5} M).

METHODS

All studies were approved by and performed in compliance with the guidelines and practices of the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Preparation of Fresh Afferent Arterioles

We used the magnetized polystyrene microsphere-sieving technique, as previously described in our laboratory, to isolate afferent arterioles (<20 μm in diameter) from 5-wk-old (90–125 g) Sprague Dawley rats maintained in the Chapel Hill Colony (8, 9). Phosphate-buffered saline (PBS), with the following composition in mM: 137 NaCl, 4.1 KCl, 0.66 KH_2PO_4, 3.4 Na_2HPO_4, 2.5 $NaHCO_3$, 1.0 $MgCl_2$, and 5 glucose, was adjusted daily to pH 7.4 at 4, 23, and 34°C. The vessel segments in PBS containing 0.1% bovine serum albumin were treated with collagenase type IV (Worthington, 374 U/mg, 3–6 μg/ml) for 18 min at 34°C. Arterioles were loaded with fura 2-AM (2–3 μM) and 0.1% bovine serum albumin for 45–60 min at 23°C in the dark. After arterioles were washed twice with PBS, the suspension was kept on ice. Ca^{2+} (1.1 mM) was added shortly before analysis of an arteriole.

Vessels were stimulated with ET-1, S6c, or IRL-1620 (10^{-7} M). The concentrations of antagonists we chose were based on our published results (8, 10, 12).

Measurement of $[Ca^{2+}]_i$

We measured $[Ca^{2+}]_i$ as previously described (8, 9). Afferent arterioles were identified by their morphology and measured diameter of 15–20 μm. As well, we required visualization of microspheres in the lumen of the afferent arteriole or in the proximal branch of an interlobular artery from which it arose, to exclude the possibility that the vessel was an efferent arteriole. The microspheres (4.0–4.5 μm) do not pass beyond the glomerular capillaries. An arteriole was centered in a small window of the optical field that was free of glomeruli or tubular fragments.

The VSMC were excited alternately with light of 340- and 380-nm wavelength from a dual-excitation wavelength Delta-Scan equipped with dual monochronometers and a chopper (Photon Technology International). After passing signals through a barrier filter (510 nm), fluorescence was detected by a photomultiplier tube. Signal intensity was acquired, stored, and processed by an IBM-compatible Pentium computer and Felix software (Photon Technology International). Background subtraction was performed in all studies. There was no interruption in the recording during the addition of reagents to the chamber. A video camera projected images of afferent arterioles onto a video monitor, permitting visualization of contraction of vessel segments.

Measurement of Superoxide with TEMPO-9-AC

The fluoroprobe TEMPO-9-AC has been used to measure superoxide production in VSMC (10, 32, 33). The fluoroprobe contains a nitroxide moiety, which quenches the fluorescence of the molecule. Superoxide or hydroxyl ions result in the loss of spin trap resonance and an increase in fluorescent emission. SOD inhibits the fluorescence response (28). We loaded afferent arterioles with TEMPO-9-AC (20 μM) for 30 min at 23°C. After the vessels were washed three times with PBS, the fluorescence signal was measured with excitation set at 361 nm and emission at 460 nm. There was a tendency for photobleaching to occur that stabilized after 40–50 s. Background subtraction was performed for each measurement.

Reagents

We purchased tempol, apocynin, 8-Br-cADPR, DPI, L-NAME, and nicotinamide from Sigma Aldrich (St. Louis, MO); ET-1, S6c, IRL-1620, BQ-123, and BQ-788 from American Peptide (Sunnyvale, CA); fura 2-AM and TEMPO-9-AC from Molecular Probes (Eugene, OR); and magnetized microspheres from Spherotech (Libertyville, IL). A-192621 was provided by Abbott Laboratories (Abbott Park, IL).

Statistics

The data are presented as means ± SE. Each data set was derived from afferent arterioles originating from at least three separate experiments, two rats (four kidneys) per experiment. Individual arterioles were studied only once and then discarded. Paired data for arterioles before and after agonist stimulation were tested with Student's paired *t*-test. Unpaired *t*-tests were employed for comparisons of responses between two groups.

RESULTS

$[Ca^{2+}]_i$ Measurements in Afferent Arterioles

$[Ca^{2+}]_i$ responses to ET-1. Afferent arterioles in Ca^{2+}-containing PBS responded to ET-1 (10^{-7} M) with a nearly immediate sharp peak increase in $[Ca^{2+}]_i$ of 303 ± 1 nM (n = 15, $P < 0.01$ vs. baseline). There was a sustained plateau of 82 ± 7 nM above baseline (27% of peak value) (Fig. 1*A*).

Tempol and apocynin diminish the $[Ca^{2+}]_i$ response to ET-1. In our previous studies of ANG II-mediated activation of NAD(P)H oxidase and production of superoxide, we suggested that superoxide increases the activity of the VSMC ADPR cyclase (10). To examine these pathways in ET-1-induced Ca^{2+} signaling, we pretreated afferent arterioles with tempol (10^{-3} M). In the presence of tempol, the peak $[Ca^{2+}]_i$ response to ET-1 was diminished to 134 ± 42 nM (56% inhibition of peak value, n = 10, $P < 0.01$ vs. ET-1 alone). The plateau was 41 ± 12 nM (30% of peak value, $P > 0.8$ vs. control ratio) (Fig. 1*B*). In the presence of apocynin (30 μM), the peak response to ET-1 was 112 ± 19 and the plateau 39 ± 7 nM (63% inhibition of peak value, n = 10, $P < 0.01$ vs. ET-1 alone, Fig. 1*C*).

The ADPR cyclase inhibitor nicotinamide decreases the $[Ca^{2+}]_i$ response to ET-1. We have previously demonstrated that nicotinamide, a specific inhibitor of ADPR cyclase, diminishes ANG II-induced responses in afferent arterioles by 66% (10). To evaluate the participation of the ADPR cyclase, cADPR pathway in ET-1-induced Ca^{2+} signaling, we pretreated afferent arterioles with nicotinamide (3×10^{-3} M). The peak increase in $[Ca^{2+}]_i$ was reduced to 115 ± 30 nM (62% inhibition, n = 6, $P < 0.01$ compared with ET-1 alone, Fig. 1*D*). The plateau level of $[Ca^{2+}]_i$ was 30 ± 5 nM.

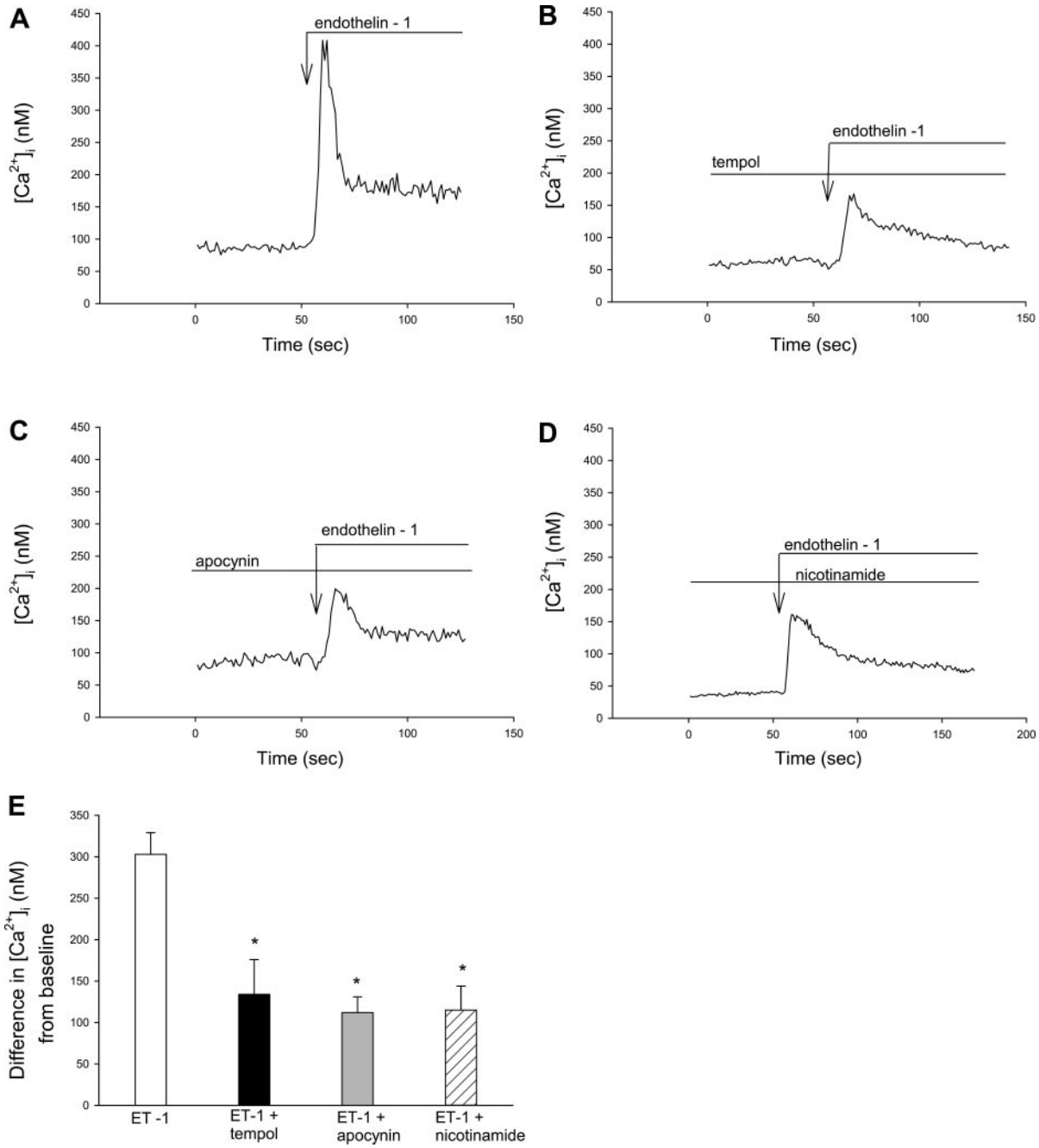

Fig. 1. Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) responses of isolated afferent arterioles to endothelin-1 (ET-1) in the presence and absence of tempol, apocynin, or nicotinamide. *A*: representative tracing of the $[Ca^{2+}]_i$ response to ET-1. *B*: typical $[Ca^{2+}]_i$ response to ET-1 in the continued presence of tempol. *C*: representative $[Ca^{2+}]_i$ response to ET-1 in the continued presence of apocynin. *D*: $[Ca^{2+}]_i$ response to ET-1 in the presence of nicotinamide. *E*: summary data of $[Ca^{2+}]_i$ responses to ET-1 (*$P < 0.01$ vs. ET-1 alone).

Summary data for the effects of tempol, apocynin, and nicotinamide are shown in Fig. 1*E*.

$[Ca^{2+}]_i$ response to ET_BR agonist S6c followed by ET-1. To investigate the presence of ET_BR-mediated $[Ca^{2+}]_i$ signaling in afferent arterioles, we employed S6c (10^{-7} M, $n = 25$). After a stable plateau level of $[Ca^{2+}]_i$ had been achieved, we added ET-1 in the continued presence of the ET_BR agonist. In general, the responses to S6c displayed a broader, more delayed (8–20 s) peak than those characteristic of ET-1. The peak $[Ca^{2+}]_i$ response to S6c was 117 ± 13 nM. These data are very similar to our previous study utilizing the ET_B agonist IRL-1620 in afferent arterioles, in which we observed a peak response of 106 ± 35 nM (9). Subsequent addition of ET-1 caused another $[Ca^{2+}]_i$ peak of 225 ± 19 nM above the S6c-stimulated plateau level (Fig. 2*A*). In our previous study of ET-1 responses following IRL-1620 activation, the peak $[Ca^{2+}]_i$ response was nearly identical, 213 ± 44 nM (9). The $[Ca^{2+}]_i$ responses of ET-1 alone are different from those following S6c activation, ($P = 0.03$), suggesting that the ET_BR has not recovered sufficiently to contribute to ET-1 activation

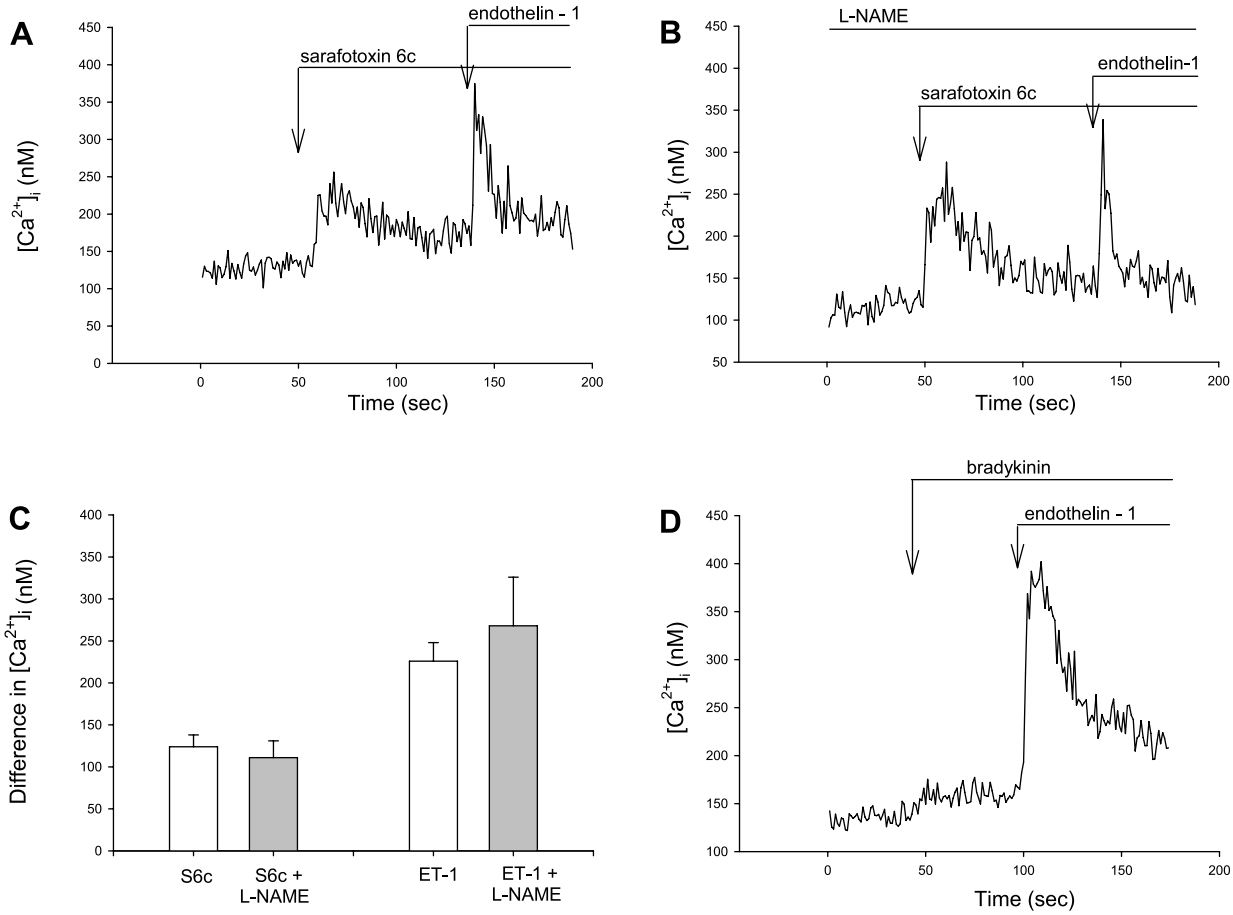

Fig. 2. $[Ca^{2+}]_i$ responses of isolated afferent arterioles to the endothelin-B receptor (ET_BR) agonist sarafotoxin 6c (S6c) followed by ET-1. *A*: representative tracing of $[Ca^{2+}]_i$ responses to S6c and the subsequent addition of ET-1 in the continued presence of S6c. *B*: representative tracing of the $[Ca^{2+}]_i$ responses in the continued presence of N^{ω}-nitro-L-arginine methyl ester (L-NAME). *C*: summary data of the $[Ca^{2+}]_i$ responses to S6c and ET-1 showing a lack of effect of L-NAME. *D*: typical tracing of the $[Ca^{2+}]_i$ response to bradykinin (10^{-6} M) followed by the addition of ET-1.

of both receptors. The sum of the S6c and ET-1 $[Ca^{2+}]_i$ responses (347 ± 24 nM) is not different from that of ET-1 alone ($P = 0.3$).

Are endothelial cells responsive to drugs administered abluminally? We have previously postulated that an influence of endothelial cells on $[Ca^{2+}]_i$ signaling is modest or absent in our afferent arteriolar preparation in which agents are added abluminally (8, 10). Furthermore, we do not know if microspheres, like air bubbles, damage endothelial cells. To document whether endothelial cell production of nitric oxide (NO) occurred during stimulation of afferent arterioles with an ET_BR agonist, we performed experiments in the presence of the NO synthase inhibitor L-NAME. In afferent arterioles pretreated with L-NAME (10^{-5} M), the $[Ca^{2+}]_i$ response to S6c was 111 ± 20 nM ($n = 7$, $P = 0.9$ vs. control). The increase in $[Ca^{2+}]_i$ following subsequent activation with ET-1 was 268 ± 58 ($P > 0.4$ vs. control, Fig. 2, *A–C*). These data strongly support the absence of endothelial cell NO production that might modify the effects of ET_AR or ET_BR stimulation of $[Ca^{2+}]_i$ signaling in our afferent arteriole preparation.

To further study the functional responsiveness of endothelial cells in our afferent arteriole preparation, we studied the effects of BK (10^{-7}-10^{-5} M) on afferent arterioles followed by addition of ET-1. Previous studies show that BK (10^{-8}-10^{-5} M) applied to the lumen stimulates an increase in $[Ca^{2+}]_i$ of 300–900 nM in a variety of vessels (26, 39, 41). In juxtamedullary afferent arterioles, administration of BK (10^{-8} M) to the perfusate caused an increase in $[Ca^{2+}]_i$ of only 57 ± 4 nM (29). VSMC of A7r5 origin had a $[Ca^{2+}]_i$ response to BK of only 10% compared with that of endothelial cells derived from bovine aorta (35).

BK (10^{-7}-10^{-5} M) caused an increase in $[Ca^{2+}]_i$ of 12 ± 2, 25 ± 12, and 27 ± 7 nM ($n = 6$, 7, and 6, respectively, not significant). The $[Ca^{2+}]_i$ responses to subsequent addition of ET-1 were 259 ± 46, 289 ± 54, and 290 ± 60 nM, none of which was different from control values (Fig. 2*D*). These data show that, when drugs are applied to the bath, there are no discernible $[Ca^{2+}]_i$ responses arising from endothelial cells. Whether or not the endothelial cells are viable after the passage of microspheres through the lumen of the vessel is not currently known.

Apocynin, nicotinamide, and 8-Br decrease ET_AR but not ET_BR $[Ca^{2+}]_i$ responses. To determine whether the production of superoxide is a consequence of both ET_AR and ET_BR activation, we pretreated afferent arterioles with apocynin before stimulation with ET_BR agonist ($n = 9$) followed by addition of ET-1, as we did above (Fig. 3*A*). In apocynin-treated arterioles, the increase in $[Ca^{2+}]_i$ after S6c was 86 ± 17 nM ($P = 0.8$ vs. control) and after ET-1, 99 ± 12 ($P = 0.01$ vs. control). These data suggest that activation of the ET_BR

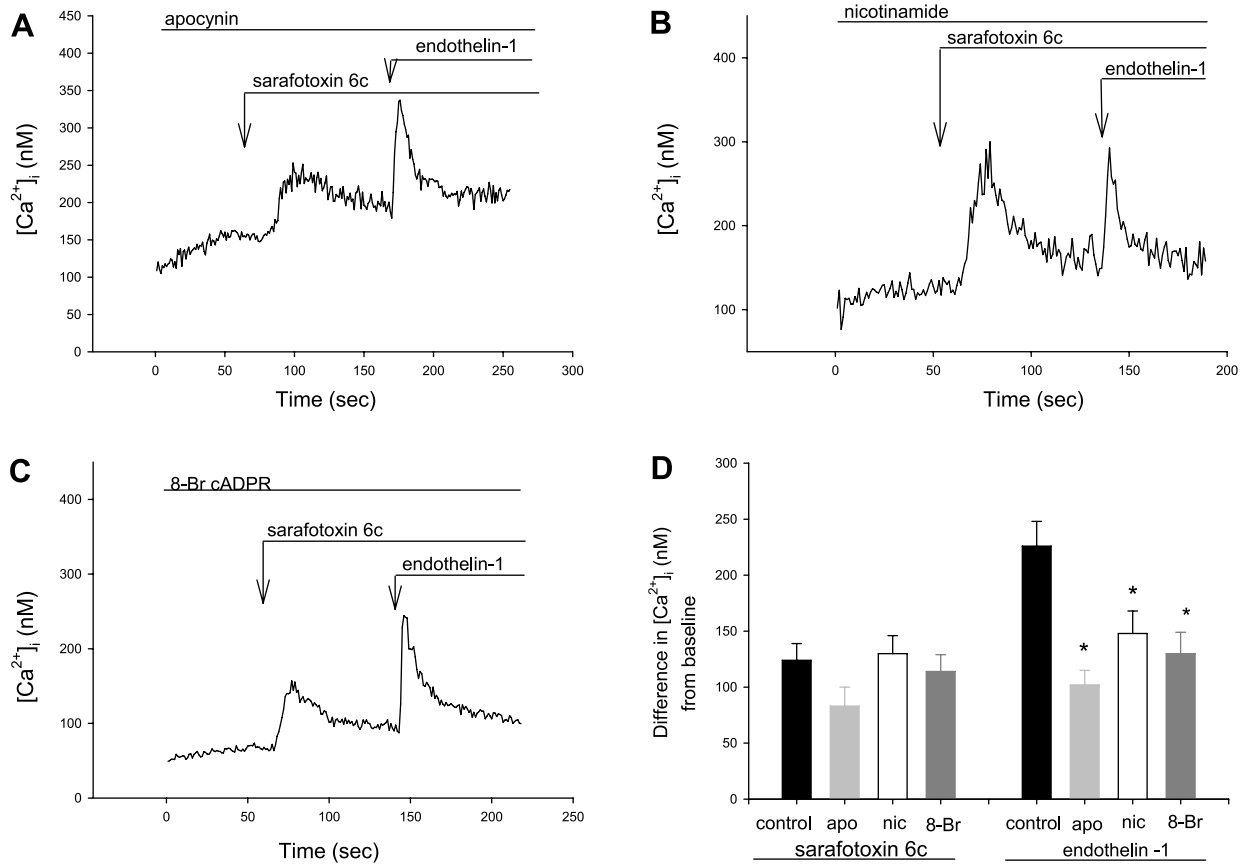

Fig. 3. Effect of blockers of superoxide generation or the adenine diphosphate ribose (ADPR) cyclase system on $[Ca^{2+}]_i$ responses of isolated afferent arterioles to S6c followed by ET-1. *A*: representative tracing of the $[Ca^{2+}]_i$ response to S6c and ET-1 in the presence of apocynin, an inhibitor of NAD(P)H oxidase. *B*: typical tracing of the effect of nicotinamide, an inhibitor of ADPR cyclase, on the $[Ca^{2+}]_i$ response to S6c and ET-1. *C*: typical tracing showing the lack of effect of 8-bromo (8-Br)-cyclic ADPR (cADPR) on the $[Ca^{2+}]_i$ response to S6c and inhibition of the response to ET-1. *D*: summary data of $[Ca^{2+}]_i$ responses to S6c and ET-1 in the presence and absence of apocynin, nicotinamide, or 8-Br demonstrating a lack of effect on S6c-induced responses, but inhibition of subsequent ET-1 responses (*$P < 0.01$ vs. control ET-1).

does not lead to significant production of superoxide via stimulation of NAD(P)H oxidase in afferent arterioles (10). In contrast, the $[Ca^{2+}]_i$ response to subsequent addition of ET-1 was reduced by 56%, just as it was in the experiments above with apocynin and ET-1 alone. In a similar fashion, we tested the ability of nicotinamide (3×10^{-3} M) to interfere with the formation of cADPR (Fig. 3*B*). In the presence of nicotinamide, the peak $[Ca^{2+}]_i$ response to S6c was 130 ± 16 nM ($n = 10$), which was not different from control. The response to ET-1 in the continued presence of nicotinamide and S6c was 148 ± 20 nM ($P < 0.01$ vs. control). To further examine the participation of the ADPR cyclase pathway in ET_BR vs. ET_AR $[Ca^{2+}]_i$ signaling, we used the specific inhibitor of cADPR, 8-Br-cADPR (10^{-4} M) (Fig. 3*C*). Responses to S6c were unaffected (114 ± 15 nM, $n = 9$, P vs. control, 0.7). In contrast, the $[Ca^{2+}]_i$ response to ET-1 in the continued presence of S6c was 130 ± 19 nM ($P = 0.02$ vs. control, 43% inhibition). Figure 3*D* summarizes the effects of these inhibitors on ET_BR and ET_AR $[Ca^{2+}]_i$ responses.

Blockade of the ET_BR. To assess the relative contributions of ET_AR and ET_BR activation to the $[Ca^{2+}]_i$ response in afferent arterioles, we pretreated the vessels with the selective ET_BR antagonist BQ-788. In the presence of BQ-788 (10^{-6} M), S6c increased $[Ca^{2+}]_i$ by only 8 ± 1 nM; subsequent addition of ET-1 caused a peak increase in $[Ca^{2+}]_i$ of 100 ± 7 nM, a value less than one-half of that in control ($n = 10$, $P < 0.01$ vs. ET-1 following S6c without BQ-788, Fig. 2*A*). The ET-1 results were not different following BQ-788 pretreatment, whether or not S6c was given after BQ-788 (111 ± 12 nM). That the ET-1 response during ET_BR antagonist treatment was about one-half of what was observed with ET_BR stimulation substantiates a complex interactions between the ET_BR and ET_AR (vide infra). We also tested BQ-788 at a lower concentration (10^{-7} M). Under these conditions, the $[Ca^{2+}]_i$ response to S6c was 66 ± 13 nM ($n = 6$, $P = 0.06$ vs. control) and to ET-1, 264 ± 44 nM ($n = 6$, Fig. 4*A*). These data confirm the effective BQ-788 concentration of 10^{-6} M.

To further substantiate the premise that blockade of the ET_BR diminishes the effect of ET-1 on the ET_AR, we utilized the specific ET_BR blocker A-192621 (3×10^{-8} M), a concentration at which inhibition of the ET_AR does not occur (38). The response to S6c was obliterated; the subsequent $[Ca^{2+}]_i$ response to ET-1 was 89 ± 18 nM ($n = 11$, $P < 0.01$ vs. control, Fig. 4*B*).

Blockade of ET_AR. We employed the specific ET_AR blocker BQ-123 (10^{-6} M) to examine the relative contribution of ET_AR activation to ET-1 responses. In the presence of BQ-123, S6c increased $[Ca^{2+}]_i$ by 120 ± 16 nM ($n = 5$, $P = 0.1$ vs. S6c control), whereas the subsequent $[Ca^{2+}]_i$ response to ET-1 was

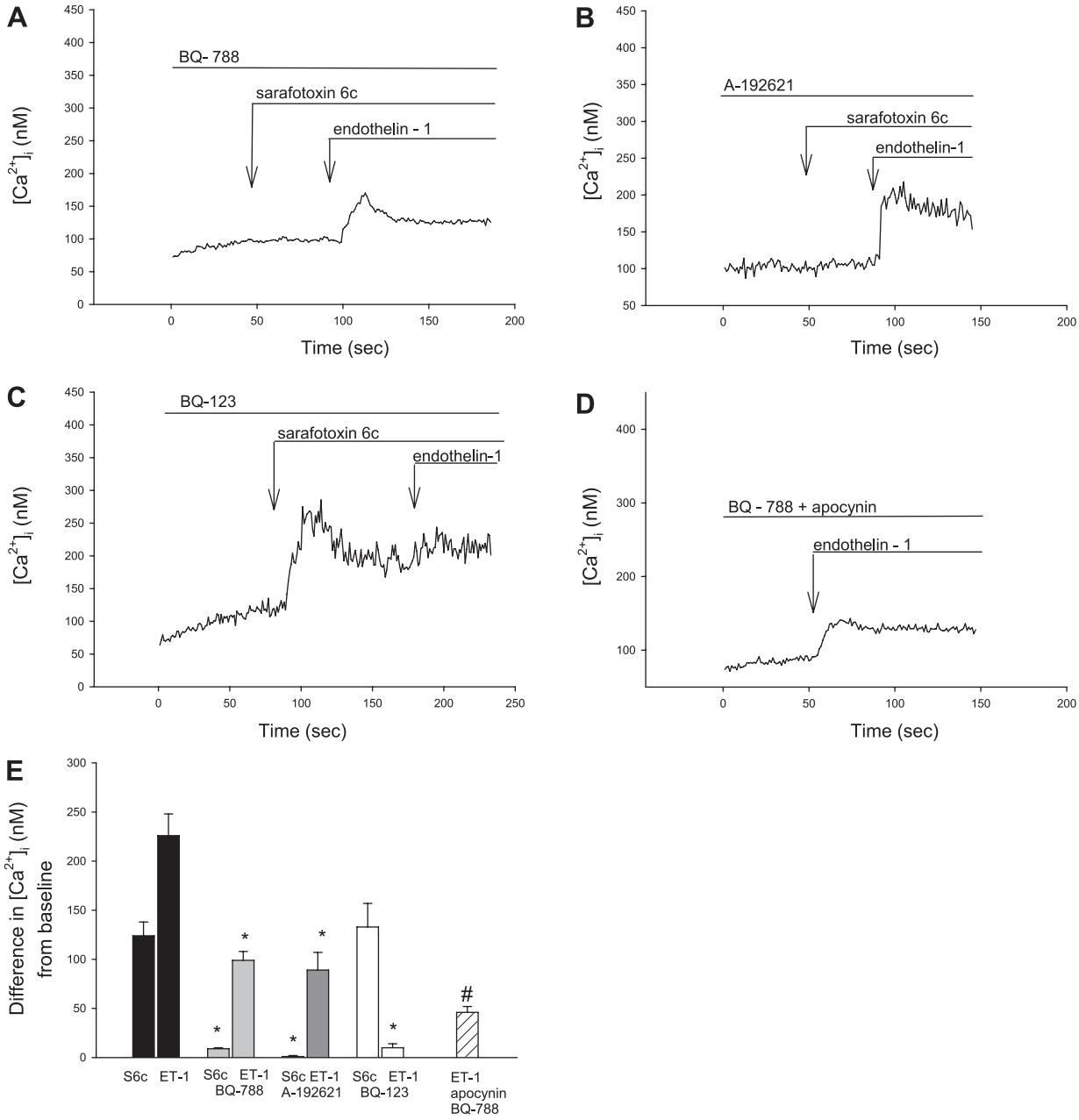

Fig. 4. Afferent arteriolar $[Ca^{2+}]_i$ responses to S6c and ET-1 in the presence of specific receptor subtype blockers. *A*: typical tracing illustrating the antagonism of ET$_B$R activation by BQ-788 and the subsequent decrease in the ET-1 $[Ca^{2+}]_i$ response. *B*: representative tracing of the effect of the ET$_B$R blocker A-192621 on the $[Ca^{2+}]_i$ response to S6c and ET-1. *C*: representative tracing of the effect of the endothelin-A receptor (ET$_A$R) blocker BQ-123 on the $[Ca^{2+}]_i$ responses to S6c and ET-1. Blockade of the ET$_A$R does not influence the response to S6c. *D*: apocynin further inhibits the $[Ca^{2+}]_i$ response to ET-1 in the presence of BQ-788. *E*: summary data. *$P < 0.01$ vs. control. #$P < 0.01$ vs. ET-1 response in presence of BQ-788.

nearly abolished (14 ± 7 nM vs. control, 225 ± 19 nM, Fig. 4*C*). These data suggest that blockade of the ET$_A$R has no effect on activation of the ET$_B$R and that the ET-1 effect following ET$_B$R stimulation is predominantly mediated by the ET$_A$R, that is, there are few, if any, unoccupied ET$_B$R available for stimulation.

ET-1 response in the presence of ET$_B$R antagonism and apocynin. To further explore the issue of receptor subtype specificity and the participation of superoxide in the $[Ca^{2+}]_i$ responses to ET-1, we pretreated afferent arterioles with both BQ-788 and apocynin. The $[Ca^{2+}]_i$ response of 46 ± 6 nM (n = 6) was about one-half the value found in the absence of apocynin ($P < 0.01$, Fig. 4*D*). As noted above, apocynin inhibited the ET-1 $[Ca^{2+}]_i$ response following stimulation with S6c by 55%. Figure 4*E* summarizes the $[Ca^{2+}]_i$ responses to S6c and ET-1 in the presence and absence of specific receptor blockers.

Measurement of Superoxide in Afferent Arterioles with TEMPO-9-AC

Effect of apocynin and DPI on ET-1-mediated production of superoxide. To confirm that stimulation of afferent arterioles with ET-1 causes the formation of superoxide, we measured

the TEMPO-9-AC fluorescence in the absence and presence of apocynin or the less selective inhibitor, DPI. ET-1 immediately increased fluorescence by 68 ± 14 arbitrary units ($n = 18$, $P <$ 0.01 vs. baseline, Fig. 5*A*). In the presence of DPI ($n = 4$) or apocynin ($n = 4$), the pooled increase in fluorescence units was 4 ± 1 arbitrary units (DPI 4.8, apocynin 3.6) ($P < 0.01$ vs. baseline and vs. ET-1 alone, 95% inhibition, Fig. 5*A*).

Effect of apocynin and DPI on ET_BR-activated superoxide generation. Because we had not previously tested the effect of ET_BR agonist IRL-1620 in experiments measuring superoxide production with TEMPO-9-AC, we studied both IRL-1620 ($n = 6$) and S6c ($n = 8$). Stimulation of afferent arterioles with S6c or with IRL-1620 caused an increase in fluorescence of 5 ± 1 and 4 ± 1 units, respectively ($P < 0.01$ vs. baseline, Fig. 5*B*). The mean increase in fluorescence with subsequent addition of ET-1 was 53 ± 15 arbitrary units. These data show that the production of superoxide is a more minor signaling pathway for ET_BR activation than is the case for ET_AR stimulation in afferent arteriolar VSMC.

DISCUSSION

We have previously provided evidence for functional ET_AR and ET_BR on $[Ca^{2+}]_i$ signaling in VSMC of fresh afferent arterioles and individual VSMC derived from preglomerular resistance vessels (9). Why renal resistance vessels have two receptor subtypes that putatively serve the same function, namely an increase in $[Ca^{2+}]_i$ to initiate vascular contraction, is not known. Redundancy in physiological signaling systems is not uncommon. However, given our recent investigations of ANG II signaling pathways and the demonstration of involvement of superoxide generation in the stimulation of the ADPR cyclase with subsequent enhancement of the $[Ca^{2+}]_i$ signal via opening of the RyR, we asked the question if stimulation of both ET_AR and ET_BR utilize the NAD(P)H oxidase and ADPR cyclase pathways (8, 10).

To our knowledge, there have been no previous in vitro studies of ET-1-mediated production of reactive oxygen species in renal resistance vessels, and, with the exception of cultured human gluteal VSMC, there are no studies of any mammalian resistance vessel or VSMC derived from it (34). As noted above, it has been proposed that conduit vessels may have only ET_AR, whereas resistance vessels have both ET_AR and ET_BR (37). Thus studies examining ET-1-stimulated formation of superoxide in large vessels would be expected to show inhibition by ET_AR but not ET_BR blockers. Investigations in cultured A-10 cells show a dose-dependent increase in superoxide production following treatment with ET-1; this effect was blocked by the ET_AR inhibitor BQ-123 (30). In aortic rings, the combination of cyclosporine and oxidized low-density lipoprotein lead to the formation of endothelin and a subsequent increase in superoxide as measured by chemiluminescence (14). BQ-123 abolishes the measured increase in ROS (14). ET-1 stimulates the formation of superoxide in vena cava VSMC, but the ET_BR agonist S6c does not (24). In primary cultures of fetal pulmonary artery VSMC, ET-1-mediated stimulation of superoxide was blocked by the ET_AR inhibitor PD156707 (36).

Based upon our work with ANG II in afferent arterioles of the rat and with ET-1 in anterior mesenteric artery of *S. acanthias*, we have developed a model for peptide agonist-mediated increases in $[Ca^{2+}]_i$ in VSMC (8, 10, 11, 12). We believe that, following G protein-coupled receptor activation, stimulation of phospholipase C, and formation of inositol 1,4,5-triphosphate (IP_3), there is a transient burst of Ca^{2+} release from the SR. The burst of Ca^{2+} can then activate calcium-induced calcium release via the RyR. The sensitivity of the RyR to Ca^{2+} is enhanced by cADPR, derived from the plasmalemmal enzyme ADPR cyclase (16). Recently, we have shown that ANG II activates VSMC NAD(P)H oxidase to increase the formation of superoxide, which, in turn, stimulates the ADPR cyclase to augment the synthesis of cADPR (10).

To examine the participation of the NAD(P)H oxidase and ADPR cyclase pathways in ET-1-stimulated afferent arterioles, we pretreated the vessel segments with tempol (a SOD mimetic), apocynin [an inhibitor of NAD(P)H oxidase assembly], or nicotinamide (an inhibitor of VSMC ADPR cyclase). Each of the three inhibitors blocked the $[Ca^{2+}]_i$ response to ET-1 by ~60%. These data suggest that a major fraction of the increase in $[Ca^{2+}]_i$ in VSMC occurs by sharing a common signaling pathway. However, the results do not give insight into the endothelin receptor subtypes involved.

In the present study, we confirm our previous findings regarding the functional presence of ET_AR and ET_BR in $[Ca^{2+}]_i$ signaling afferent arterioles (9). We show that, after

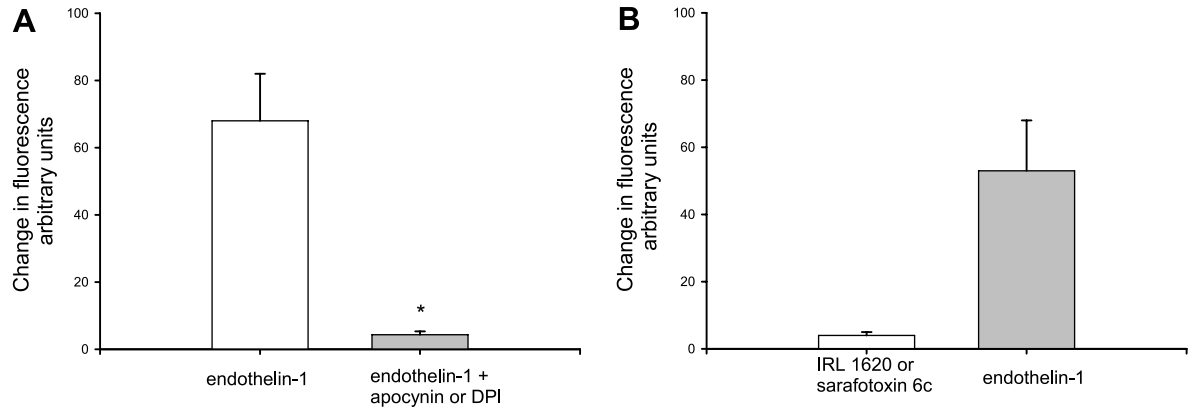

Fig. 5. Measurement of superoxide with TEMPO-9-AC. *A*: summary data showing that apocynin and diphenyl iodonium (DPI) inhibit ET-1-induced superoxide production by 95% (*$P < 0.01$). *B*: summary data of the production of superoxide by S6c or IRL-1620 followed the addition of ET-1 in the continued presence of the ET_BR agonists.

stimulation of VSMC with an ET_BR agonist, subsequent addition of ET-1 causes a new peak $[Ca^{2+}]_i$ response that is about twice that of the ET_BR agonist (117 and 225 nM, respectively). The sum of the two peak $[Ca^{2+}]_i$ responses is not different from the response to ET-1 alone. That the $[Ca^{2+}]_i$ response to ET-1 following S6c in the presence of the ET_AR blocker BQ-123 is only 18 nM (vs. 225 nM in its absence) suggests that there is not a significant number of residual ET_BRs available for stimulation. Hence, one can conclude that the $[Ca^{2+}]_i$ response to ET-1 after S6c (in the time frame of these experiments) primarily represents ET_AR activation.

One might predict that the $[Ca^{2+}]_i$ response to ET-1 following ET_BR agonist activation would be similar to that following blockade of the ET_BR with the ET_BR inhibitors BQ-788 or A-192621. Such is not the case in our studies. Whereas the $[Ca^{2+}]_i$ response to ET-1 after S6c stimulation was 225 ± 19 nM, the response in the presence of BQ-788 (10^{-6} M) was less than one-half that (100 ± 7 nM, $P < 0.01$). A lower concentration of BQ-788 (10^{-7} M) only blocked the S6c response by ~50%, suggesting that BQ-788 binding to the ET_AR is not occurring (20). Similarly, A-192621 totally abolished the response to S6c, but the subsequent response to ET-1 was only 89 ± 18 nM.

Our previous $[Ca^{2+}]_i$ studies in afferent arterioles utilizing IRL-1620 and BQ-788, both at concentrations of 10^{-7} M, showed inhibition of the IRL-1620 response but not of the subsequent ET-1 response (9). Why there was blockade of the ET_BR agonist response in that study compared with the current one is unknown. In every other comparison of IRL-1620 and S6c in afferent arterioles from our laboratory, the responses are similar.

The results in the present study showing the difference in ET_AR responses following stimulation vs. blockade of the ET_BR suggest the possibility that activation of the ET_BR may result in ET_BR-ET_AR heterodimerization and, therefore, in augmentation of the subsequent Ca^{2+} signaling cascade (15, 17, 18). Studies in nonvascular tissue demonstrate that the ET_BR may stimulate the formation of active heterodimers (15, 18). Such an interaction might explain why a combination of both ET_AR and ET_BR antagonists is required to completely inhibit ET-1-induced afferent arteriolar constriction (19). A recent study in pulmonary arteriolar VSMC suggests that ET_BR-ET_AR heterodimerization may take place (29a). In vivo studies also suggest that there are ET_AR/ET_BR interactions that have an effect on renal vascular reactivity (21).

When afferent arterioles are pretreated with the specific ET_AR antagonist BQ-123, the $[Ca^{2+}]_i$ response to S6c is not different from control. As expected, the subsequent response to ET-1 is all but obliterated (14 ± 7 nM). Thus it appears that the $[Ca^{2+}]_i$ response to ET_BR stimulation is independent of ET_AR activation, in contrast to our data suggesting an influence of the ET_BR on ET_AR activation.

Pretreatment of afferent arterioles with apocynin has no significant effect on the $[Ca^{2+}]_i$ response to S6c, but does reduce the magnitude of the response to the subsequent addition of ET-1 to 99 ± 12 nM (56% inhibition). These data suggest that, under the conditions of this experimental protocol, ET_BR are not discernibly involved in the NAD(P)H oxidase/superoxide pathway. Our studies showing a lack of an effect of the NO synthase inhibitor L-NAME on $[Ca^{2+}]_i$ signaling make it unlikely that endothelial cell production of NO (and production of peroxynitrite) can account for these findings. Furthermore, we show that BK (10^{-7}-10^{-5} M) causes only a small (<10% of ET-1 response) change in $[Ca^{2+}]_i$, likely representing the effect of BK on VSMC rather than endothelial cells (26, 35, 39, 41).

That nicotinamide and 8-Br-cADPR did not alter the $[Ca^{2+}]_i$ responses to S6c, but did inhibit those to subsequent administration of ET-1 in afferent arterioles lends additional support to the premise that it is primarily ET_AR but not ET_BR stimulation that results in the activation of ADPR cyclase and sensitization of the RyR.

As we did with ET-1 alone, we measured the formation of superoxide with TEMPO-9-AC following agonist stimulation of afferent arterioles. Because we had not tested the ET_BR agonist IRL-1620 with TEMPO-9-AC, we employed this agent as well as S6c. The measured increase in superoxide production in response to S6c and IRL-1620 stimulation is only 5 ± 1 and 4 ± 2 arbitrary units, respectively. These values are significantly different from baseline ($P < 0.01$). Subsequent addition of ET-1 causes the formation of 53 ± 15 units of superoxide, a value not different from that achieved by stimulation with ET-1 alone. These data, derived from addition of TEMPO-9-AC to the bath, strongly suggest that ET_AR but not ET_BR activation plays a major role in the formation of superoxide in afferent arteriolar VSMC. Furthermore, our data with nicotinamide and 8-Br-cADPR (vide supra) suggest that superoxide is activating the ADPR cyclase to form cADPR, which then sensitizes the RyR to Ca^{2+} to augment the $[Ca^{2+}]_i$ response (10). The results suggest that, in afferent arteriolar VSMC, ET_BR stimulation primarily activates Ca^{2+} signaling pathways other than the NAD(P)H, superoxide, ADPR cyclase pathway. It is possible that the small amount of superoxide produced in response to ET_BR activation (<10% compared with ET-1 receptor stimulation) is insufficient to dimerize ADPR cyclase and cause increased production of cADPR.

Our in vitro results in afferent arterioles advance our knowledge about mechanisms of endothelin-mediated $[Ca^{2+}]_i$ signaling in VSMC. The VSMC appear to be uninfluenced by ET_BR activation of endothelial cells and the production of NO. There is no connection to a glomerulus or an efferent arteriole, and there are no contributing effects of tubule-derived products of ET_BR stimulation. Possible effects of tubuloglomerular feedback are absent. We await in vivo studies to further our understanding regarding these interactions.

When one studies the evolutionary history of endothelin, it is apparent that the presence of both ET_AR and ET_BR in animals is a more recent biological event. In tilapia, torpedo fish, lizard, toad, fundulus, and dogfish shark, there is evidence for ET_BR but not ET_AR; however, in the eel, turtle, and sea lamprey, only ET_AR is identified (7, 13, 23, 31, 42). The appearance of both receptor subtypes is first noted in the chicken embryo heart (22). It remains mysterious why there has been species difference with regard to having one receptor or the other. In mammals, vascular endothelial cells exclusively express ET_BR, but both ET_BR and ET_AR are present on VSMC of resistance vessels, whereas conduit vessels have only ET_AR (37). The present study shows that ET_AR activation results in the formation of superoxide from NAD(P)H oxidase, but that stimulation of isolated afferent arteriolar VSMC with S6c or IRL-1620 (10^{-7} M) results in an amount that is only 5% of that value. In contrast, in peritubular smooth muscle cells of rat

testis, the ET_BR appears to operate exclusively via activation of the RyR, but the ET_AR utilizes both the IP_3 receptor and the RyR (1). In celiac ganglion neurons and PC-12 cells, S6c stimulates the production of superoxide, and BQ-788 inhibits this effect by ~70% (3). In what other ways ET_AR and ET_BR activation differ in their physiological responses will be of interest to pursue in the future.

In summary, we confirm that $[Ca^{2+}]_i$ signaling in afferent arteriolar VSMC involves both ET_AR and ET_BR and show for the first time that ET_AR activation rapidly increases the formation of superoxide via NAD(P)H oxidase that accounts for ~60% of the immediate $[Ca^{2+}]_i$ response. In contrast, ET_BR activation appears to result in only minor (<10%) increases in superoxide production. Our studies with nicotinamide and 8-Br-cADPR suggest that ET-1 (and primarily ET_AR), like ANG II, causes the activation of VSMC ADPR cyclase through the production of superoxide and that cADPR, by sensitizing the RyR to Ca^{2+}, augments the increase in $[Ca^{2+}]_i$ initially generated by IP_3-receptor-mediated release of Ca^{2+} from the SR. The functional consequences of ET_AR and ET_BR interactions await further investigation.

GRANTS

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