Sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibition prevents endothelin A receptor antagonism in rat aorta

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Tosun M, Erac Y, Selli C, Karakaya N. Sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibition prevents endothelin A receptor antagonism in rat aorta. Am J Physiol Heart Circ Physiol 292: H1961–H1966, 2007. First published December 15, 2006; doi:10.1152/ajpheart.00298.2006.—This study tested whether sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase regulates the ability of endothelin receptor antagonist to inhibit the endothelin-1 constriction. The endothelin A receptor antagonist BQ-123 (1 μM) completely relaxed constriction to 10 nM endothelin-1 in endothelium-denuded rat aorta. Challenge with cyclopiazonic acid (10 μM), a sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor, during the plateau of endothelin-1 constriction enhanced the constriction by ~30%. BQ-123 relaxed the endothelin-1 plus cyclopiazonic acid constriction by only ~10%. In contrast, prazosin (1 μM), an α-adrenergic receptor antagonist, still completely relaxed the 0.3 μM phenylephrine constriction in the presence of cyclopiazonic acid. Verapamil relaxed the endothelin-1 plus cyclopiazonic acid constriction by ~30%, whereas Ni\(^{2+}\) and 2-aminoethoxydiphenyl borate, nonselective cation channel and store-operated channel blockers, respectively, completely relaxed the constriction. These results suggest that lowered sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase activity selectively decreases the ability of endothelin receptor antagonist to inhibit the endothelin A receptor. The decreased antagonism may be related to the opening of store-operated channels and subsequent greater internalization of endothelin A receptor.

BQ-123; vascular smooth muscle; store-operated calcium; caveola

THE ROLE OF STORE-OPERATED CALCIUM (SOC) IN THE REGULATION OF RECEPTOR-OPERATED VASCULAR TONE IS SOMewhat controversial. The apparent complexity is partly due to the presence of SOC in both cell types found in blood vessels, endothelial cells, and vascular smooth muscle cells. Depletion of agonist-sensitive Ca\(^{2+}\) stores in either cell type results in elevation of cytosolic Ca\(^{2+}\) concentration through SOC channels (SOCCs) (21). Elevation of Ca\(^{2+}\) in endothelial cells activates endothelial nitric oxide synthase, yielding to endothelium-dependent vascular smooth muscle relaxation via nitric oxide (17, 38, 39). On the other hand, in the absence of intact endothelium, SOCCs also mediate Ca\(^{2+}\) elevation in vascular smooth muscle (1, 13, 26, 27, 29, 31, 33). Store-operated channels may be responsible for nonspecific cation entry during any inositol 1,4,5-trisphosphate (IP\(_3\))-producing receptor stimulation and coupled to vascular contraction in the presence of certain agonists, such as endothelin (ET) (see Ref. 16 for review).

ET, one of the most potent vasoconstricting peptides released from endothelial cells (35), exerts its effects through two types of sarcolemmal G protein-coupled receptors, ET\(_A\) and ET\(_B\) (3, 25). ET\(_A\) subtype is profoundly expressed in the vascular smooth muscle and is responsible for the constricting effects of ET-1. ET\(_B\), on the other hand, is also present at membranes of endothelial cell and vascular smooth muscle cell nucleus, counteracting the pressor effects of ET-1 and preventing nuclear Ca\(^{2+}\) overload (7).

More than a decade ago, ET\(_A\) receptors were shown to be localized in caveolae in the absence of its ligand (8). A structural unit of caveola, caveolin-1, appears as a potential regulator of either normal or pathological smooth muscle cell functions (28). Caveolar structures cluster signaling molecules through caveolin and PDZ (postsynaptic density protein, Drosophila disc large tumor suppressor, Zo-1 protein) domain proteins in a spatially confined subsarcolemmal region to make protein-protein interactions more rapid and efficient (34). A recent study (20) also suggests that internal PDZ ligand motif seems to regulate efficient recycling of the ET\(_A\) endothelin receptor. Later, growing evidence suggested that caveolin-1 regulates the interaction of ET-1 with its receptors (37). Moreover, increased vascular reactivity to ET-1 in hypercholesterolemic animals and human subjects supports the role of caveolar localization of ET\(_A\), especially in pathophysiological conditions (14, 24). Most recently, caveolin-1-deficient aortic smooth muscle cells show abnormal ET-mediated signal transduction (10). Decreased contractility to ET-1 but not to dopolarization or α-adrenergic receptor (α-AR) stimulation upon caveolar disruption also suggests differential membrane dynamics, depending on preferential colocalization of membrane proteins in vascular smooth muscle (6).

TRPC1, a mammalian homologue of D. melanogaster transient receptor potential (trp) channel subtype, is reportedly responsible for SOC entry (5, 32). Although ET receptor colocalization/functional dependence with TRPC1 (5, 32) and recycling have been already reported (6), the role of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) blockade leading to SOC entry in the caveola vicinity is not known. SOC may be coupled to contraction by downstream receptor signaling cascade, possibly due, in part, to activation of PKC, which may also be restricted to the same compartments (2, 29). Our results suggest a simple model whereby SOC facilitates internalization of ET\(_A\) receptors into subsarcolemmal compartments that cannot be accessed by BQ-123. Experimental blockade of SERCA in this study may only mimic a diseased state or treatment (i.e., corticosteroid) causing downregulation of SERCA expression. Therefore, the purpose of this study was to test our hypothesis that SERCA blockade regulates the ability of ET...
receptor antagonist to inhibit the ET-1-mediated contraction of rat thoracic aorta.

METHODS

All animal experiments were approved by the Institution’s Committee on Animal Use in Research and Education and adhered to the principles adopted by the American Physiological Society. Male Sprague-Dawley rats (300–350 g) were asphyxiated with CO2, and the thoracic aorta segments were isolated, immediately placed in physiological salt solution (PSS; Krebs-Ringer bicarbonate solution with 10 μM indomethacin to eliminate endogenous prostanoid synthesis), and cleaned of surrounding fatty tissue (22). Each aorta was cut into 3-mm-long circular segments, and the endothelium was removed by gently rubbing the intimal surface with stainless steel wire. Ring preparations were mounted between two stainless-steel hooks in organ baths, each containing 25 ml of Krebs solution (37°C) gassed with 95% O2-5% CO2. The upper wire was connected to an isometric force-displacement transducer (FDT 10-A; Commat, Turkey), which was mounted on a displacement unit allowing a fine adjustment of tension. The preparations were allowed to equilibrate for 45 min in PSS. During this period, the organ baths were washed with fresh (37°C) PSS solution every 15 min. After 45 min, each ring was gradually stretched in 0.5-g increments to the previously established optimal point of the resting tension (20 mN) for rat aorta. Once at the optimal length, the segments were allowed to equilibrate for 30 min before experimentation. Deendothelialization was confirmed by the lack of acetylcholine-induced relaxation during phenylephrine (PE) challenges before the test protocol.

All organ bath measurements were recorded with a digital data acquisition system (Biopac Systems). Force was normalized to cross-sectional area [force (in mN)/area (in mm²) = (change in force × circumference)/2 × wet wt).

Statistics. All values are expressed as means ± SE; n represents the number of animals used. Statistical significance was evaluated using Student’s t-test (in case of two groups) or Newman-Keuls test for multiple comparisons. P < 0.05 was considered significant.

Chemicals. All standard laboratory chemicals were from Sigma-Aldrich Chemical. Stock solutions and subsequent dilutions were prepared in distilled water. Only cyclopiazonic acid (CPA) was dissolved in DMSO, the concentration of which was <0.1%, a critical level that causes no vascular relaxation.

RESULTS

The overall experimental protocol in this study was constructed on the basis of the observation that 10 μM CPA did not affect the resting tone in the presence of indomethacin (10 μM) in deendothelialized rat thoracic aorta (present study and Ref. 29).

ET-1 concentration (10 nM), causing ~25% of maximal effect (E_max) was used throughout the experimental protocol. The reason was that ET-1 showed quite steep concentration-response relationship [negative logarithm of 50% effective concentration (pD2) = 7.57 ± 0.28; n = 3], yielding maximum response in 1 log unit in rat aorta in the absence of endothelium.

Fig. 1. Effect of BQ-123 (BQ), an endothelin A (ETA) receptor antagonist, on ET-1-induced contraction. A: representative tracings. B: summarized data from experiments in A. F/CSA, force normalized to cross-sectional area. **P < 0.01, Student’s t-test (paired); n = 4.

Fig. 2. Effects of cyclopiazonic acid (CPA) and 2-aminoethoxydiphenyl borate (2-APB) on ET-1 contraction. A: sample tracings. B: summarized data from experiments in A. **P < 0.01 vs. BQ; n = 4.
CPA-enhanced ET-1 contractions were not abolished by BQ-123. Although BQ-123 (1 μM) abolished 10 nM ET-1 contractions (Fig. 1), it only partially inhibited ET-1 contractions in the presence of CPA (Figs. 2 and 3). CPA (10 μM) that did not elicit force in the absence of endothelium, significantly enhanced 10 nM ET-1 plateau contractions (Figs. 2 and 3).

The ETA receptor antagonist BQ-123 was then added to see whether enhanced force was due to uncompensated agonist-induced elevation of intracellular Ca²⁺ concentration ([Ca²⁺]i) by the removal of sarcoplasmic reticulum (SR)-buffering capacity through CPA-mediated SERCA inhibition. In some arteries, BQ-123 showed no inhibition at all. The remaining tone was abolished by a purported SOC entry blocker, 2-aminoethoxydiphenyl borate (2-APB; Fig. 2).

We also tested the partial contribution of voltage-operated Ca²⁺ channels (VOCCs) and nonselective cation channels (NSCCs) in CPA-enhanced ET-1 response remaining after BQ-123. For this purpose, a VOCC blocker, verapamil (1 μM), and a NSCC blocker, Ni²⁺ (0.1, 0.3, and 1 mM), were added sequentially. Ni²⁺ abolished the remaining contraction following verapamil and BQ-123 (Fig. 3). Verapamil did not modulate ET receptor antagonism.

Relative contributions of VOCCs and NSCCs were tested on 10 nM ET-1 contractions. The high level of 10 nM ET-1 contrac-
tion was inhibited almost 40\% by 1 \( \mu \)M verapamil; 1-(2-trifluoromethylphenyl)imidazole (TRIM), a purported blocker of SOC entry, had only a slight inhibition on the high level of ET-1 contraction, whereas Ni\(^{2+}\) abolished the remaining tone (Fig. 4).

We also tested whether VOCCs are involved in CPA-induced contractions in the absence of any IP\(_3\)-producing receptor activation. For this purpose, a PKC activator, phorbol dibutyrate (PDB), was added at a low concentration that does not elevate force and cytosolic Ca\(^{2+}\) concentration but is enough to couple SOC to contraction on the basis of our earlier observation (29). The substantial amount of force observed following CPA was partially sensitive to the VOCC blocker verapamil while remaining abolished by the NSCC blocker Ni\(^{2+}\) (Fig. 5).

**CPA presence did not alter \( \alpha \)-AR antagonism in PE-induced contractions.** To investigate whether the decrease in antagonistic effect also occurs for other receptor-mediated responses, a non-peptide vasoconstrictor agonist, PE, was used. The rationale for using PE was based on the suggestion that \( \alpha_1 \)-AR could be an essential component of TRPC6 (12), a candidate transient receptor potential channel subtype for SOC entry. Another reason for using PE was that \( \alpha_1 \)-ARs may not participate in caveola-associated receptor trafficking (9). Therefore, a similar experimental procedure was also performed with PE.

Contractions induced by submaximal concentrations (300 nM) of PE (pD\(_2\) = 7.32 \( \pm \) 0.04, \( n = 3 \)) were sensitive to the \( \alpha_1 \)-AR selective antagonist prazosin (1 \( \mu \)M) (Fig. 6). CPA (10 \( \mu \)M) further enhanced submaximal PE responses (Fig. 7). Unlike other observations done with ET-1, PE-induced responses were abolished by prazosin regardless of CPA presence (Fig. 7).

**DISCUSSION**

The present study reports a novel observation that BQ-123 does not abolish ET-1-induced contraction in the presence of a SERCA inhibitor, CPA. On the other hand, complete reversal of CPA-enhanced PE responses by the \( \alpha \)-AR antagonist prazosin, suggested to us that CPA-induced Ca\(^{2+}\) elevation may alter ET\(_\alpha\) receptor-mediated responses, which we earlier reported its predominant role in subarachnoid hemorrhage-induced basilar artery spasm (40).

CPA-induced responses during receptor activation may result from either coupling of SOC to contraction or removal of buffering capacity of superficial SRs (30). A conflicting observation to the latter is that 10 nM PDB, a PKC activator, had no apparent effect on force/[Ca\(^{2+}\)]\(_i\), whereas the addition of CPA...
drastically elevated the resting tone, which was abolished by sequential additions of verapamil and Ni\textsuperscript{2+} (29). Moreover, KCl-induced contractions were not enhanced by CPA (2, 29), suggesting that lack of SR buffering capacity may not be accounted for by enhanced force.

**Effects of CPA on agonist-induced force and the source of Ca\textsuperscript{2+} in deendothelialized rat aorta.** Although 10 μM CPA was shown to elevate [Ca\textsuperscript{2+}]i in vascular tissues (see Ref. 16 for review), there was no force generated by CPA alone in endothelium-denuded aorta in the presence of indomethacin. This is also supported by an observation in intact mouse aorta that CPA caused transient contraction, which was lost by indomethacin pretreatment or thromboxane A\textsubscript{2} receptor blockade (18). In the present study, 10 μM CPA enhanced contractions induced by ET-1 and PE (Figs. 2, 3, and 7), possibly through activation of PKC present within the same subsarcolemmal compartment where SOC is elevated (Fig. 5 and Ref. 29).

Previously, we have shown that CPA-induced [Ca\textsuperscript{2+}]i elevation was not sensitive to verapamil, whereas it was completely inhibited by the NSCC blocker Ni\textsuperscript{2+} in a concentration-dependent manner (29). Furthermore, ET-1-induced force generation was only partially sensitive to verapamil, whereas it was abolished by Ni\textsuperscript{2+} addition in rat aorta (Fig. 4), rabbit basilar artery, and rat caudal artery (6, 40), suggesting the predominant involvement of NSCCs in ET receptor activation. Verapamil sensitivity could be explained by the membrane depolarization induced by massive SOC entry and reverse activation of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Predominant NSCC type in ET-1 responses could be SOC since the verapamil-insensitive portion of ET-1 contraction was abolished by the purported blocker 2-APB (Fig. 2). SOCCs could be activated by internalization of ET-1 or ET-1/ET\textsubscript{R} complex (23), followed by a direct interaction of ET-1 and/or ET\textsubscript{R} with SERCA pumps (4).

**Effects of CPA on receptor blockade.** A drastic decrease in ETA receptor antagonism on CPA-enhanced ET-1 contractions (Figs. 2 and 3) suggests possible inaccessibility of BQ-123 to ETA receptor. Earlier, the loss of BQ-123 effect when administered following ET-1 in rat ventricular cardiomyocytes despite the use of techniques that block internalization led the authors to comment on the “quasi-irreversible” feature of ET receptor activation due to a proposed refractory population of ET-1/ET\textsubscript{R} complex (11). However, in rat aorta (present study), the decrease in ET\textsubscript{R} antagonism by BQ-123 was not a time-dependent event, because it inhibited ET-1 steady-state contraction even after 90 min in the absence of CPA (included in cumulative data, Fig. 1). The decrease in the inhibitory effect of BQ-123 was only evident after CPA administration. Although membrane impermeability of BQ-123 favors the idea of ET-ET\textsubscript{R} internalization, we cannot exclude the possible presence of some receptor population that somewhat becomes refractory to BQ-123 during SOC entry. However, this still may not explain the profound loss of receptor antagonism.

This observation is somewhat specific to ET-1 effect because of the fact that PE-induced contractions were always abolished by prazosin regardless of CPA administration (Fig. 7). This observation is also in agreement with that reported earlier for rat thoracic aorta (15). Disruption of caveolar structures by cyclodextrin-induced cholesterol depletion has been shown to be correlated with decreased contractility to ET-1 but not to depolarization and α-AR activation (9). The observation of unaffected KCl contraction in the presence of significant levels of CPA-induced [Ca\textsuperscript{2+}], is also consistent with the idea of spatially distinct localization of SOC entry and that of VOCC (29). In addition, the decrease in ET receptor antagonism does not seem to be due to Ca\textsuperscript{2+} entry via VOCC because it persisted after verapamil (Fig. 3B).

Colocalization of TRPC1, a transient receptor potential channel subtype reportedly responsible for SOC entry, with ET\textsubscript{A} receptor in caveolar structures (6) further suggests that SOC entry induces ET\textsubscript{A} receptor internalization. Therefore, it seems plausible that ET-1 facilitates internalization of caveola with its receptor. Internalized ET-1 and/or activated ET\textsubscript{A} may further inhibit SERCA (4). Internalization of ET\textsubscript{A} receptor may account for the drastic decrease in ET receptor antagonism. Preferential caveolar localization yields recycling because some functional ET receptor population slowly reappears on cell surface independent of de novo protein synthesis (15). However, this process does not seem to be operational for adrenergic receptors since they do not meet the criteria necessary to interact with caveolin-1 (19).

In conclusion, this study suggests that SOC entry activated by SERCA blockade enhances ET receptor internalization, leading to prolonged vascular contractility. This might have a clinical impact in that an undesired vasospasm may even be more detrimental in disease states in which vascular smooth muscle SERCA isoform is downregulated similar to that shown in cardiomyocytes of failing hearts (36).

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