Simultaneous Measurement of Force and Calcium Uptake During Acetylcholine-Induced Endothelium-Dependent Relaxation of Rabbit Thoracic Aorta

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This study was designed to determine whether the endothelium-derived relaxing factor induced by acetylcholine (1 μM) in rabbit thoracic aorta inhibits agonist-induced calcium mobilization, specifically calcium influx. Force generated in rings of rabbit thoracic aorta by norepinephrine (1 μM) was measured under isometric conditions. At the appropriate time during 1 μM acetylcholine-induced relaxation of 1 μM norepinephrine-contracted rabbit thoracic aorta, the rings were pulse-labelled with calcium-45 to measure calcium influx. When measured in this fashion, 1 μM acetylcholine decreased the 1 μM norepinephrine-induced increase in calcium influx. This effect was eliminated by removal of the endothelium and by atropine (1 μM), but not by indomethacin (14 μM). Acetylcholine (1 μM) also blocked the 60 mM potassium-chloride-induced increase in calcium influx without dramatically affecting force. The phasic contraction produced by norepinephrine (1 μM) with 2 mM lanthanum pretreatment, which is caused by release of intracellular calcium, was inhibited by acetylcholine (1 μM) in a fashion similar to 1 μM nitroglycerin. The tonic contraction produced by norepinephrine (1 μM) after depletion of the agonist-releasable pool of intracellular calcium, which is thought to be due to calcium influx, was depressed by acetylcholine (1 μM). These data suggest that endothelium-derived relaxing factor relaxes 1 μM norepinephrine-contracted rings of rabbit thoracic aorta by decreasing calcium entry and by producing an extracellular calcium-independent relaxant effect. (Circulation Research 1987;60:31-38)

Acetylcholine (ACh) relaxes isolated rings of norepinephrine-contracted rabbit thoracic aorta. Relaxation is rapid and appears to involve an endothelium-derived relaxing factor (EDRF) that is released in response to activation of endothelial cell muscarinic receptors. Several other agents produce the same response in aorta and other arteries. It has recently been suggested that EDRF stimulates the production of guanosine 3',5'-cyclic monophosphate (cGMP) in vascular smooth muscle, which in turn mediates vasodilatation. Since the extent of active force generated by vascular smooth muscle is calcium-dependent, EDRF may cause relaxation by decreasing myoplasmic calcium.

The present investigation was designed to determine whether endothelium-dependent relaxation of isolated rabbit thoracic aorta is caused by an alteration in the ability of norepinephrine (NE) to mobilize calcium for contraction. Results suggest that the mechanism by which ACh induces endothelium-dependent relaxation may only partially involve blockade of calcium influx. Other potential mechanisms are discussed.

Materials and Methods

Tissue Preparation

Adult male New Zealand white rabbits were killed by cervical fracture and immediately exsanguinated. The chest of each animal was opened and the entire descending thoracic aorta was rapidly but gently removed and placed in warm (35-37°C), oxygenated physiological saline solution (PSS, composition in mM: NaCl, 140.0; KCl, 5.0; MgCl₂, 1.0; dextrose, 10.0; CaCl₂, 1.5; HEPES, 5.0; pH 7.3). All reagents except HEPES were ACS certified (Fisher Scientific). HEPES was Sigma grade (Sigma Chemical Co., St. Louis, Mo.). Doubly glass-distilled water was used in all solutions. Aortas were cleaned of adhering tissue and cut into vascular rings approximately 3 mm long to be used for the measurement of force or for the simultaneous measurement of force and calcium influx. Before equilibration in the contractile or calcium uptake apparatus and immediately after dissection, the vascular rings were preequilibrated for 60 minutes in warm, oxygenated PSS to stabilize the tissue. Unless otherwise indicated in the section on "Results," each ring was labelled with radioisotope or contracted only once. Vascular rings were studied on the day they were obtained.

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Isometric Force and Calcium Influx

To record isometric force alone, each vascular ring was placed across two stainless steel wires within individual chambers each containing 20 ml of oxygenated PSS. The lower wire was secured firmly to an oxygenator while the upper wire was connected to a Statham UC2 force transducer. To record both force and calcium influx, 4 rings were placed across their own wires within a single chamber containing 20 ml of oxygenated PSS. Each vascular ring was adjusted to maintain a resting (baseline) force of 1 g during an equilibration period lasting 60 minutes. Following equilibration, tests described in the section on "Results" were begun. Any change in force from baseline constituted active force. The active force generated by each ring was recorded over time on a Gould chart recorder (Model 2800) and was expressed as mg force/mg wet tissue weight.

The procedures used to measure calcium influx have been previously reported in detail. These procedures are based on those originally described by Meisheri et al with some modifications. To measure calcium influx, vascular rings were radiolabelled for 3 minutes with Ca (2.5 Ci/ml, New England Nuclear, Boston, Mass.), then washed for exactly 90 minutes in 60 ml of vigorously stirred, oxygenated, ice-cold, calcium-free PSS containing 2 mM EGTA. Following the wash period, each vascular ring was blotted on filter paper, weighed on an electronic balance (Mettler, Model AE163), and incubated overnight at room temperature in 2 ml of 7.5 mM EDTA. Liquid scintillation cocktail for aqueous samples was then added and each ring was counted (Beckman scintillation counter, Model LS6800). Calcium uptake was calculated as the EGTA-resistant Ca fraction (cpm/kg) divided by the specific activity of the Ca-containing PSS (cpm/µmole calcium).

The relatively short labelling period of arterial rings measures primarily the unidirectional influx of calcium. This conclusion is based on previously reported data showing a linear relation between calcium uptake and time over the labelling period for both basal and agonist conditions in rabbit thoracic aorta (RTA). To test the validity of this conclusion for the present study, an experiment was conducted to determine the relation between calcium uptake and time in rings exposed to 1 µM NE plus 1 µM ACh. Rings, prepared as previously described for calcium influx experiments, were exposed to NE at zero time, exposed to ACh at 3 minutes, and exposed to Ca at 3.5 minutes. Rings were removed for washing and subsequent scintillation analysis 1.5, 3.0, and 5.0 minutes subsequent to the initiation of Ca labelling.

Drugs and Solutions

The following drugs were dissolved in glass-distilled water or warm PSS (6.8% ethanol and 6.8% PEG for nitroglycerin) and injected into the PSS bathing vascular rings in a volume 0.1% that of the bathing media: acetylcholine chloride (ACh, Sigma Chemical Co., St. Louis, Mo.), atropine sulfate (Merck Sharp & Dohme, West Point, Penn.), lanthanum chloride (La, Sigma), nitroglycerin (TNG, American Critical Care, Philadelphia, Penn.), (-)-norepinephrine bitartrate (NE, L-arterenol, Sigma), indomethacin (Merck Sharp & Dohme). All drug solutions were made fresh before each experiment and were kept on ice. To depolarize arteries with high KCl a solution was made by replacing 60 mM NaCl with 60 mM KCl.

PSS, not yet adjusted to pH 7.3 and excluding calcium, was made as a stock solution and kept refrigerated. Calcium (1.5 mM, calcium-containing PSS) or ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (2 mM, EGTA, Fisher, calcium-free PSS) was added to the stock solution, and the result was adjusted to pH 7.3 with NaOH and HCl on the morning of the experiment.

Statistical Analyses

Data are shown as means ± SEM, and n represents the number of aortas. The null hypothesis was examined by Analysis of Variance and Duncan's multiple range test or by the nonpaired Student's t test and was rejected at p < 0.05. Calculations and statistical analyses were performed with the aid of a microcomputer (Eagle Inc.).

Results

Effect on Calcium Mobilized by Norepinephrine

ACh (1 µM) caused a rapid and relatively transient relaxation when added to rings of RTA precontracted by 1 µM NE (Figure 1). The concentration of ACh used throughout these experiments (1 µM) was chosen because it elicited the maximal vasodilator effect on tissues precontracted with 1 µM NE. At 10 µM, ACh began to act as a vasoconstrictor. To determine whether ACh produces an inhibition of calcium influx that is concomitant with its relaxant effect, Ca was injected into the medium bathing vascular rings that were attached to a force transducer for monitoring isometric force rather than into the medium bathing nonstretched, freely hanging rings, as is commonly done when measuring calcium uptake into vascular tissue. By maintaining muscle length constant, potential length-dependent changes in calcium influx can be avoided. Using this technique, it was ensured that calcium influx was measured during the relaxation phase of the transient ACh vasodilation.

Basal calcium influx in rings of RTA stretched to 1 g
Figure 2. Effect of short incubation times on cellular accumulation of $^{45}$Ca in rings of rabbit thoracic aorta. Uptake was linear through 3 minutes (correlation coefficient of linear regression = 0.94). Numbers in parentheses represent number of different rings (one ring per rabbit) studied at each timepoint. The open symbol at the 5-minute timepoint was excluded from the regression analysis since it deviated significantly from the linear relation.

Passive force was $20.2 \pm 1.1$ μmoles/kg tissue wt/3 min, $n = 8$. Both 1 μM NE and 60 mM KCl significantly elevated calcium influx above the basal level in stretched rings of RTA (μmole/kg/3 min: NE 32.4 ± 2.0, $n = 16$; KCl 39.1 ± 2.2, $n = 7$; both $p<0.05$ compared to basal calcium influx).

In a separate experiment, calcium uptake was measured as a function of time in rings exposed to 1 μM NE plus 1 μM ACh to determine if calcium influx rate was uniform over the pulse-labelling period. The results (Figure 2) show that under these conditions, calcium influx rate was uniform over the 3-minute $^{45}$Ca pulse and equaled 5 μmolecules Ca$^{2+}$/kg tissue wt/min. At 5 minutes, influx rate was reduced, suggesting that additional calcium handling mechanisms (i.e., calcium efflux) had been activated.

Figure 3 illustrates a comparison in the ability of ACh and the nonspecific calcium antagonist La$^{3+}$ to alter calcium influx and force produced by NE in rings of RTA. NE-contracted RTA relaxed rapidly after exposure to 1 μM ACh compared to 2 mM La$^{3+}$, and both agents decreased NE-stimulated calcium influx. La$^{3+}$, however, produced a greater inhibition of NE-induced calcium influx than did ACh, although at the time calcium influx was measured ACh had relaxed rings of RTA by approximately 62%, whereas La$^{3+}$ had relaxed rings by only about 35%. When calcium influx was measured 10–13 minutes after the addition of ACh to NE-contracted RTA, rather than 2–5 minutes later as in Figure 3A, calcium influx was no longer depressed (μmole/kg/3 min: NE control 38.3 ± 2.0; NE + ACh 38.8 ± 2.8). Thus, it appears that the ability of ACh to cause both relaxation and a decrease in stimulated calcium influx is transient.

Nitro-vasodilators elevate cellular cGMP, and it is believed that this intracellular messenger activates relaxation. Rapoport and Murad have recently shown that ACh-induced endothelium-dependent relaxation of rat aorta may be mediated by cGMP. In the present study, 1 μM TNG relaxed NE-contracted RTA rapidly, and like ACh, caused a small but statistically significant decrease in NE-stimulated calcium influx (Figure 4). Contraction of RTA produced by NE are caused by both influx of extracellular calcium and release of intracellular calcium. Since ACh inhibited calcium influx in NE-contracted RTA, a test was devised to determine whether ACh could inhibit force generated solely by calcium influx. Rings of RTA were contracted for 10 minutes by NE in calcium-free PSS containing 2 mM EGTA. A subsequent contraction could not be produced unless calcium was added to the solution. This contraction was presumably caused by an increase in cytosolic calcium brought about solely by calcium influx. The contraction produced by NE-stimulated calcium influx was greatly attenuated by 1 μM ACh (Figure 5).
Effect on nitroglycerin (TNG) on the force (line tracings) and calcium influx (vertical bars) produced by 1 μM NE in rings of rabbit thoracic aorta. Arrow indicates when TNG was added. Rectangle enclosing 45Ca indicates when the vascular rings were radiolabelled. Force and calcium influx were measured in the same rings. *p<0.01, †p<0.05.

To determine whether ACh can also relax the transient NE contraction that can be produced when influx of extracellular calcium is prevented, ACh was added to rings of RTA at the peak of the transient contraction produced by NE in the presence of La3+. ACh caused an inhibition in NE-generated force within 1 minute (Figure 6A). Thus, the normal slow decline from peak force during a NE contraction in the presence of La3+ was accelerated by ACh. For a comparison, the vasodilator TNG produced a response similar to that produced by ACh (Figure 6B), whereas the addition of more La3+ did not accentuate the normal decline in force from peak (Figure 6C). The inhibition by ACh of the force generated by NE when calcium influx was blocked by La3+ suggests that ACh may cause an increase in calcium sequestration or efflux.

Figure 4. Effect of nitroglycerin (TNG) on the force (line tracings) and calcium influx (vertical bars) produced by 1 μM NE in rings of rabbit thoracic aorta. Arrow indicates when TNG was added. Rectangle enclosing 45Ca indicates when the vascular rings were radiolabelled. Force and calcium influx were measured in the same rings. *p<0.01, †p<0.05.

Effect on KCl-Induced Calcium Influx
KCl contractions are more resistant to inhibition by ACh than NE contractions.3 A comparison was made in this study between ACh and La3+ in their ability to cause relaxation of a KCl contraction (Figure 7B). ACh produced only a small, statistically nonsignificant (ANOVA) but reproducible inhibition in force and inhibited KCl-induced calcium influx to a level that was still significantly above basal influx. La3+, at both 2 and 5 mM, produced a slow relaxation, as it does when added to rings contracted by NE. At 5 mM, La3+ greatly inhibited calcium influx. The mean KCl-induced influx produced in the presence of 2 mM La3+ was depressed, although this was not statistically significant.

Endothelium, Atropine, and Indomethacin
It is well documented that ACh-induced relaxation of NE-contracted RTA is endothelium-dependent, is sensitive to muscarinic receptor blockade, and is not mediated by prostaglandins (for review, see Furchgott4). To determine whether the same is true for the ACh-inhibition of NE-induced calcium influx, vascular rings of RTA contracted by 1 μM NE were challenged with 1 μM ACh after 1) removal of the endothelium by rubbing, 2) incubation with 1 μM atropine, and 3) incubation with 14 μM indomethacin. The rings were radiolabelled with 45Ca for 3 minutes, 1–4 minutes after addition of ACh.

Removal of the endothelium prevented the relaxation normally produced by ACh and prevented the decrease in NE-stimulated calcium influx (Figure 8A). Likewise, atropine completely eliminated both the re-
relaxation and decrease in calcium influx produced by ACh (Figure 8B). The cyclooxygenase inhibitor indomethacin did not block the ACh-induced relaxation or inhibition of calcium influx (Figure 8C), suggesting that both relaxation and calcium influx blockade were not mediated by cyclooxygenase products of arachidonic acid.

**Discussion**

It has long been known that ACh contracts most isolated vascular preparations but often causes vasodilation in vivo. A principal reason for these apparently contradictory findings was delineated by Furchgott who showed that the endothelium is vitally important in modulating vascular reactivity to ACh and that most in vitro vascular preparations (primarily helical strips) have damaged (nonfunctional) endothelium.  

It has recently been demonstrated that ACh and other agents can relax NE-induced, as well as other autacoid-induced, contractions produced in several arteries via an endothelium-dependent mechanism.  

The mechanism by which ACh and other agents that modulate endothelial cell activity causes relaxation of precontracted vascular preparations is not fully understood. The existence of an EDRF has been demonstrated, but its identity has eluded clarification. It has recently been suggested that endothelium-dependent relaxation is mediated by an elevation in the level of cGMP produced in vascular smooth muscle cells.

Cytoplasmic calcium levels in vascular smooth muscle are regulated by calcium channel activity, membrane-bound calcium pumps, and intracellular sequestering mechanisms. Since the level of cytosolic calcium directly determines the extent of force generated by vascular smooth muscle, ACh-induced endothelium-dependent relaxation may be the result of an
Figure 8. Effect of removal of the endothelium (Panel A), and addition of atropine (Panel B) or indomethacin (Panel C) on the ability of ACh to inhibit NE-induced force (line tracings) and calcium influx (vertical bars) in rings of rabbit thoracic aorta. Arrows indicate when ACh was added. Rectangle enclosing 45Ca indicates when the vascular rings were radiolabelled. Force and calcium influx were measured in the same rings. *p<0.01, tp<0.05.

Alteration in agonist-induced calcium mobilization, specifically, a decrease in calcium influx. The present study was designed to test this hypothesis. Results suggest that EDRF causes a moderate reduction in NE-induced calcium influx and that another relaxing mechanism may be equally important in decreasing vascular tone. In addition, this study demonstrates that ACh and TNG act in a similar fashion.

NE mobilizes both intracellular and extracellular calcium to cause contraction in the smooth muscle of RTA.16,17 Initiation of contraction is thought to be primarily the result of an increase in cytosolic calcium brought about by the release of intracellularly bound calcium.24 This store of calcium is of a limited size and requires calcium from an extracellular source for replenishment.21,24 Maintenance of force is the result of NE-induced calcium influx.17 The level of sustained tone can be reduced by exclusion of calcium from the bathing medium or by calcium blockers.17,25 In this study, 1 μM ACh caused a fast and relatively transient relaxation of vascular rings of RTA precontracted by 1 μM NE. When calcium influx was measured early enough during the relaxation, NE-induced calcium influx was significantly inhibited by ACh. This was not true if calcium influx was measured at a later time, presumably because the vasodilator effect was offset by ACh’s known direct vasoconstrictor effect.26

The decrease in NE-stimulated calcium influx produced by ACh was endothelium-dependent, since disruption of endothelial cells by rubbing6 caused ACh to lose its ability to vasodilate and reduce calcium influx. Atropine, but not indomethacin, blocked the reduction in NE-stimulated calcium influx produced by ACh. The same was true for the ACh-induced decrease in NE-induced tone. These data suggest that activation of endothelial cell muscarinic receptors caused the production of EDRF, as outlined by Furchgott.6 In addition, these data indicate that the EDRF may decrease vascular tone in part by decreasing agonist-induced calcium influx.

ACh did not cause as great an inhibition of calcium influx as the nonspecific calcium antagonist La3+ even though it produced a greater percent relaxation. In addition, the relaxation produced by ACh was much faster than that produced by La3+ (Figure 3). TNG produced a rapid, strong relaxation and a weak inhibition of NE-stimulated calcium influx in a manner similar to ACh. TNG was found by Itoh et al27 to enhance calcium extrusion from vascular smooth muscle cells, while producing only a minimal, nonsignificant inhibition of unstimulated calcium influx. These data suggest that the relaxation produced by ACh was not solely the result of calcium influx blockade.

These results prompted us to investigate the ability of ACh to relax the transient NE-induced contraction that can occur in the absence of extracellular calcium, as well as the sustained contraction that can be produced solely as a result of calcium influx after depletion of the agonist-releasable intracellular calcium pool. ACh significantly attenuated, but did not eliminate, the NE-induced contraction produced after depletion of the agonist-releasable intracellular calcium pool. This reinforces the contention that one action of EDRF is to produce some calcium influx blockade.

In order to determine the effect of ACh on the extracellular calcium-independent contraction produced by NE, ACh was added to vascular rings when the force produced by NE in the presence of 2 mM La3+ was maximal, and any relaxation produced was compared to the slow decline in force from the maximum level
that normally occurs. ACh produced a rapid and strong relaxation that was comparable to that produced under the same conditions by 1 mM TNG, despite the findings that release of EDRF appears to be calcium-dependent. Addition of more La³⁺ at the time of maximum force did not produce additional relaxation. These data suggest that ACh can relax NE-contracted RTA through a mechanism that is distinct from calcium influx blockade in vascular smooth muscle, implying that EDRF can accelerate the removal of calcium from the cytosol of vascular smooth muscle cells by causing an increase in the kinetics or the affinity for calcium of certain proteins involved in calcium sequestration and extrusion. An inhibition of NE-induced release of intracellular calcium by EDRF is less likely, since ACh was added at the peak of the NE contraction produced in the presence of La³⁺, and the calcium-release phenomenon should have been largely ended by this time. Whether EDRF-induced relaxation occurs partially through a direct modulation of the contractile proteins is unknown, and participation in this complex regulatory scheme cannot be ruled out. Since both TNG-like compounds and EDRF are known to elevate cGMP in vascular smooth muscle and cause rapid relaxation, these data imply that EDRF may exert both its extracellular calcium-independent and dependent relaxing effects by elevating cGMP.

ACh does not relax KCl-contracted rings of RTA to any great extent (Figure 6) but produces a significant reduction in KCI-stimulated calcium influx. These data support the contention that an action of EDRF is to decrease stimulus-induced calcium influx in vascular smooth muscle. Since NE and KCl appear to activate distinct calcium channels in RTA, EDRF may act nonspecifically to decrease calcium entry. In summary, isolated rings of RTA that were contracted by 1 mM NE showed an increase in calcium influx above the basal level, and 1 μM ACh significantly depressed this NE-induced increase in calcium influx concomitantly with a decrease in NE-generated tone. The ability of ACh to decrease NE-stimulated calcium influx was dependent on an intact endothelium, was mediated by muscarinic receptors, and was not caused by cyclooxygenase products. The calcium blocker effect produced by ACh is therefore attributed to the action of EDRF. Calcium influx blockade by EDRF could not, however, completely explain the ability of ACh to relax precontracted RTA, since relaxation occurred in the presence of the calcium influx blocker La³⁺. Further work on the effect of EDRF on calcium mobilization may help gain an insight into the mechanisms by which the endothelium modulates vascular smooth muscle tone.

Acknowledgments

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