Relaxation of Porcine Coronary Artery to Bradykinin
Role of Arachidonic Acid
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Abstract Bradykinin-induced relaxation of precontracted, porcine coronary artery (PCA) rings is mediated by distinctly different endothelial relaxing factors depending on the contractile agent used. Thus when contracted with KCl, bradykinin-induced relaxation of PCA rings is mediated solely by nitric oxide (NO), whereas when contracted with the thromboxane mimetic U46619, a small component of the relaxation is attributable to NO and a large component is attributable to a non-NO mechanism that is independent of cyclooxygenase activity. We hypothesized that the non-NO component was mediated by arachidonic acid (AA) or by a non-cyclooxygenase product of AA metabolism. Bradykinin-induced relaxations of PCA rings precontracted with U46619 in the presence of indomethacin (10 μmol/L) were moderately attenuated by the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L), whereas when precontracted with KCl, L-NAME abolished the relaxations. AA produced endothelium-dependent relaxations of rings precontracted with U46619 that were unaffected by L-NAME, whereas AA did not relax rings precontracted with KCl. In rings precontracted with U46619, in the presence of L-NAME and indomethacin the phospholipase inhibitors quinacrine (50 μmol/L) and 4-bromophenacyl bromide (10 μmol/L) attenuated bradykinin- but not AA-induced relaxations. Inhibitors of both lipoxygenase (BW 755c [100 μmol/L] and nafazatrom [20 μmol/L]) and cytochrome P-450 (proadifen [10 μmol/L] and clotrimazole [10 μmol/L]) pathways did not eliminate bradykinin- or AA-induced relaxations, although clotrimazole partially attenuated AA-induced relaxations. These findings suggest that bradykinin-induced relaxation of PCA rings is mediated by AA through a mechanism that is not dependent on cyclooxygenase, lipoxygenase, or cytochrome P-450 pathways. (Hypertension. 1994;23[part 2]:976-981.)

Key Words • endothelium-derived relaxing factor • coronary artery • arachidonic acid • bradykinin

The concept of the vascular endothelium as a fundamental participant in circulatory control is rapidly evolving.1,2 In addition to several vasoconstricting agents,3-5 at least two vasorelaxing substances of vascular endothelial origin have been identified: prostaglandin I₂ (PGI₂),6 a product of cyclooxygenase-mediated arachidonic acid metabolism, and nitric oxide (NO),7,8 or a closely related compound,9 which is produced by the action of NO synthase on L-arginine. More recently, endothelium-dependent relaxations of several vascular tissues have been reported to occur through a mechanism(s) distinct from one involving either cyclooxygenase or NO synthase.10-13 Thus relaxation of isolated porcine coronary arterial (PCA) rings to bradykinin, for example, persisted despite inhibition of both cyclooxygenase and NO synthase.13-15 It was proposed that this relaxation was mediated by an endothelium-derived hyperpolarizing factor.16

Bradykinin activates phospholipase A₂,17,18 purportedly through receptor-mediated increases in intracellular calcium.19 The activation of phospholipase A₂ results in hydrolysis of tissue phospholipids, in turn releasing fatty acids, including arachidonic acid.19,20 Arachidonic acid may in turn be reacylated21 or metabolized further through three known pathways, ie, the cyclooxygenase pathway to prostaglandins and thromboxanes, the lipoxygenase pathway22 to leukotrienes and oxygenated fatty acids, and the cytochrome P-450 monooxygenase pathway22 to epoxides and oxygenated fatty acids. Several of the non-cyclooxygenase-mediated products of arachidonic acid metabolism are capable of relaxing blood vessels.24-26

In view of the finding that bradykinin produces relaxation of PCA rings through a mechanism that is in large part independent of NO synthase and cyclooxygenase, we hypothesized that bradykinin-induced relaxation of PCA rings is mediated by arachidonic acid itself or through a product of arachidonic acid metabolism that is independent of cyclooxygenase activity.

Methods

Pig hearts were obtained from a local slaughterhouse; placed immediately into ice-cold modified Krebs-Ringer bicarbonate (KRB) solution containing (in mmol/L) NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, Na-EDTA 0.026, and glucose 11.1; and transported to the laboratory. The right coronary artery was dissected, cleansed of all adherent fat and connective tissue, and cut into rings 3 to 5 mm wide. Each ring was mounted on two stainless-steel posts, one fixed and the other movable. The latter post was attached to an isometric force transducer (model FT-03, Grass Instrument Co) coupled to a polygraph (model 7, Grass) for continuous recording of ring tension. The rings were suspended in water-jacketed (37°C) organ baths (Radnoti Glass
Technology, Inc) containing 10 or 20 mL KRB solution and continuously aerated with 95% O₂-5% CO₂. Basal ring tension was increased incrementally. With each increment, the ring was contracted with KCl (60 mmol/L) until a maximal contraction was identified. The rings were maintained at the basal tension that produced a maximal contraction (range, 9 to 12 g; mean, 10.0±0.1 g) for the remainder of the experiment. Indomethacin (10 μmol/L) was in the KRB solution throughout all experiments. When used, N^6-nitro-L-arginine methyl ester (L-NAME) was introduced into the bath 45 minutes prior to the second application of arachidonic acid in rings contracted with U46619 (Fig 1A). In rings pretreated with L-NAME (n=7), AA relaxations were reduced (Fig 1A); however, significant relaxation persisted, suggesting the presence of a NO-independent mechanism of vasorelaxation. Endothelium dependency was determined in U46619-precontracted rings that were untreated, pretreated with N^6-nitro-L-arginine methyl ester (L-NAME) (100 μmol/L), or mechanically denuded of endothelium (n=9); B, BK relaxations of KCl-precontracted rings, untreated or pretreated with L-NAME (n=7); C, AA relaxations of U46619-precontracted, endothelium-intact (E+) rings in the presence of L-NAME (n=7); U46619-precontracted, endothelium-denuded (E−) rings (n=6); and KCl-precontracted, endothelium-intact (E+) rings (n=5). D, GTN-induced relaxations of endothelium-intact rings precontracted with U46619 or KCl (n=5). Indomethacin (10 μmol/L) was included in all experiments. Results are expressed as mean±SEM. *P<.05, untreated vs L-NAME-pretreated or endothelium-denuded (BK data) rings; U46619-precontracted, endothelium-intact rings vs U46619-precontracted, denuded rings or KCI-precontracted, intact rings (AA data).

**Chemicals**

Bradykinin (acetate salt), arachidonic acid (sodium salt), U46619, indomethacin, L-NAME, quinacrine, BPB, proadifen, clotrimazole, and cromokalim were purchased from Sigma Chemical Co. Nitroglycerin was purchased from American Regent Laboratories, Inc. BW 755c and nafazatrom were the gifts of The Wellcome Research Laboratories and Miles Laboratories, Inc, respectively. Indomethacin, BPB, clotrimazole, and cromokalim were dissolved in ethanol; U46619 was dissolved in methanol; and nafazatrom was dissolved in dimethyl sulfoxide (DMSO). Final bath concentrations of ethanol and DMSO did not exceed 0.1%. All other chemicals were dissolved in distilled water.

**Statistical Analysis**

All values are given as mean±SEM. Differences between values were analyzed by Student’s t tests for paired and unpaired data, as appropriate. Differences between mean values of multiple groups were analyzed by analysis of variance, with a least significant difference test applied if the F ratio was significant. Values of P<.05 were considered statistically significant.

**Results**

**Responses to Bradykinin**

Bradykinin (0.3 to 100 nmol/L) produced concentration-dependent relaxations of PCA rings precontracted with U46619 (Fig 1A). In rings pretreated with L-NAME (100 μmol/L), responses to bradykinin were reduced (Fig 1A); however, significant relaxation persisted, suggesting the presence of a non-NO-mediated mechanism of vasorelaxation. Endothelium dependency of the response was demonstrated by the observation that mechanical disruption of the endothelium abol-

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Figure 1. Plots of relaxation responses to bradykinin (BK), arachidonic acid (AA), and nitroglycerin (GTN) in porcine coronary artery rings. A, BK relaxations were determined in U46619-precontracted rings that were untreated, pretreated with N^6-nitro-L-arginine methyl ester (L-NAME) (100 μmol/L), or mechanically denuded of endothelium (n=9); B, BK relaxations of KCl-precontracted rings, untreated or pretreated with L-NAME (n=7); C, AA relaxations of U46619-precontracted, endothelium-intact (E+) rings in the presence of L-NAME (n=7); U46619-precontracted, endothelium-denuded (E−) rings (n=6); and KCl-precontracted, endothelium-intact (E+) rings (n=5). D, GTN-induced relaxations of endothelium-intact rings precontracted with U46619 or KCl (n=5). Indomethacin (10 μmol/L) was included in all experiments. Results are expressed as mean±SEM. *P<.05, untreated vs L-NAME-pretreated or endothelium-denuded (BK data) rings; U46619-precontracted, endothelium-intact rings vs U46619-precontracted, denuded rings or KCl-precontracted, intact rings (AA data).
FK3. Plots of effects of the phospholipase inhibitors quinacrine and 4-bromophenacyl bromide (BPB) on bradykinin (BK), arachidonic acid (AA), and nitroglycerin (GTN) relaxations of porcine coronary artery rings precontracted with U46619 in the presence of $N^\bullet$-nitro-L-arginine methyl ester and indomethacin. A, BK relaxations were determined before (control) and after treatment with quinacrine (50 jtmol/L) (n=6). B, AA relaxations before (control) and after quinacrine (n=5). C, GTN relaxations before (control) and after quinacrine (n=5). D, BK relaxations were determined before (control) and after treatment with BPB (10 jtmol/L) (n=6). E, AA relaxations before (control) and after BPB (n=6). F, GTN relaxations before (control) and after BPB (n=6). Results are expressed as mean±SEM. *P<.05, control vs treatment with quinacrine or BPB.

Figures

Fig 1A: Plots of effects of the phospholipase inhibitors quinacrine and 4-bromophenacyl bromide (BPB) on bradykinin (BK), arachidonic acid (AA), and nitroglycerin (GTN) relaxations of porcine coronary artery rings precontracted with U46619 in the presence of $N^\bullet$-nitro-L-arginine methyl ester and indomethacin. A, BK relaxations were determined before (control) and after treatment with quinacrine (50 jtmol/L) (n=6). B, AA relaxations before (control) and after quinacrine (n=5). C, GTN relaxations before (control) and after quinacrine (n=5). D, BK relaxations were determined before (control) and after treatment with BPB (10 jtmol/L) (n=6). E, AA relaxations before (control) and after BPB (n=6). F, GTN relaxations before (control) and after BPB (n=6). Results are expressed as mean±SEM. *P<.05, control vs treatment with quinacrine or BPB.

Discussion

The findings of the present study are consistent with previous studies14,15 in which endothelium-dependent relaxations of the PCA produced by bradykinin were reported to be resistant to the application of either
methylene blue or hemoglobin (both of which inhibit NO-mediated vasorelaxation\(^{13}\)) and to the administration of either NO synthase or cyclooxygenase inhibitors. Furthermore Cowan and Cohen\(^{15}\) demonstrated that increased cyclic GMP concentrations in PCA rings after exposure to bradykinin were prevented by \textsuperscript{N}^-\textsuperscript{monomethyl}-L-arginine or methylene blue and that cyclic AMP (which increases in response to PGI\(_2\)) was not stimulated by bradykinin. Therefore endothelium-dependent relaxation of the PCA occurs through a process that is distinct from either of the two previously identified endothelium-derived factors, NO and PGI\(_2\).

Although L-NAME had only a minor effect on bradykinin-induced relaxation of PCA rings contracted with U46619, it abolished bradykinin relaxation of rings contracted with KCl. These findings are in agreement with those of Cowan and Cohen\(^{15}\) and Nagao and Vanhoutte.\(^{16}\) One interpretation of these results is that the major component of bradykinin-induced relaxation of PCA rings contracted with U46619 occurs through a mechanism that is not affected by inhibition of NO synthase but is blocked by KCl, which opposes hyperpolarization. This relaxing mechanism might then result from membrane hyperpolarization and its attendant effects on cytosolic free calcium. Agents such as bradykinin, which produce endothelium-dependent relaxation of the PCA, have also been reported to hyperpolarize PCA endothelial and smooth muscle cells.\(^{13,27}\)

Hyperpolarization is believed to occur secondary to an enhanced efflux of potassium ions from vascular smooth muscle cells after the activation of potassium channels. The resultant hyperpolarization then is believed to close voltage-dependent calcium ion (Ca\(^{2+}\)) channels, thereby reducing Ca\(^{2+}\) entry into the smooth muscle cells and resulting in vasorelaxation.\(^{28}\) Hyperpolarization of PCA smooth muscle cells in response to bradykinin was reported to be dependent on the presence of the endothelium.\(^{10}\) Thus the possibility that the relaxant effect of bradykinin on PCA rings is mediated by the release of a hyperpolarizing factor is a reasonable hypothesis.

In the present study we hypothesized that the non-NO-, cyclooxygenase-independent relaxation of PCA rings produced by bradykinin was mediated by arachidonic acid or through products of a non-cyclooxygenase pathway of arachidonic acid metabolism. This hypothesis was formulated in view of the findings that bradykinin receptor activation activates phospholipases in a variety of tissues, resulting in the release of arachidonic acid\(^{17}\); arachidonic acid contributes to bradykinin-induced relaxation of isolated canine coronary artery rings through its conversion to cytochrome P-450 metabolites\(^{24}\); arachidonic acid\(^{29}\) as well as several metabolites of arachidonic acid can activate potassium channels on smooth muscle cells\(^{30-32}\); and potassium channel activation was reported to be a mechanism by which 11,12-epoxyeicosatrienoic acid, a product of epoxygenase-mediated arachidonic acid metabolism, produced relaxation of isolated cat cerebral arteries.\(^{33}\) As a first step in addressing this hypothesis, we characterized responses of PCA rings to the administration of arachidonic acid. In the presence of indomethacin, arachidonic acid produced concentration-dependent relaxations of rings contracted with U46619, which were not affected by pretreatment with L-NAME but were markedly attenuated by removal of the endothelium. In contrast, when the rings were contracted with KCl, arachidonic acid did not produce relaxation. These data suggest that arachidonic acid primarily relaxes PCA rings through an endothelium-dependent mechanism that does not require NO but is inhibited by excess K\(^+\), suggesting that the relaxation may result from potassium channel activation. This finding is consistent with the hypothesis that arachidonic acid (or one of its metabolites) effects the relaxation of PCA rings in response to bradykinin. The endothelium-dependent relaxation produced by bradykinin does not appear to be mediated by free arachidonic acid acting directly on vascular smooth muscle cells, however, because arachidonic acid–induced relaxation was also found to be endothelium dependent.

If arachidonic acid does mediate bradykinin-induced relaxation of the PCA, then agents that inhibit bradykinin-induced arachidonic acid release from tissue phospholipids would also be expected to attenuate bradykinin relaxation responses. Arachidonic acid can be liberated from cellular membranes through hydrolysis by phospholipase A\(_2\) or by the sequential actions of
phospholipase C and diglyceride lipase. Quinacrine, which inhibits phospholipases by interfering with the substrate-enzyme interface, and BPB, which inhibits phospholipases by alkylating a histidine moiety near the active site of the enzyme, are nonselective agents that have been reported to inhibit phospholipase C as well as phospholipase A₂. These agents would be expected to inhibit bradykinin-induced relaxation if this response is dependent on the release of arachidonic acid from tissue stores. Indeed, bradykinin-induced relaxation was inhibited by both quinacrine and BPB, whereas relaxations to nitroglycerin and arachidonic acid were not attenuated by either compound. Furthermore BPB did not attenuate relaxation produced by the ATP-dependent potassium channel activator cromakalim. These observations suggest that quinacrine and BPB may have inhibited bradykinin-induced relaxation by interfering with a proximal step in the signal transduction process, ie, arachidonic acid release, as opposed to a distal one, ie, arachidonic acid metabolism. These findings are consistent with our hypothesis that arachidonic acid, or one of its metabolites, mediates bradykinin-induced relaxation of the PCA. To evaluate the possibility that a lipoxygenase or cytochrome P-450 product of arachidonic acid metabolism mediates the bradykinin-induced endothelium-dependent relaxation of PCA rings, responses to bradykinin and arachidonic acid were studied in the presence and absence of inhibitors of both enzymatic pathways. The lipoxygenase inhibitor nafazatrom did not affect either bradykinin- or arachidonic acid–induced relaxation, whereas the combined cyclooxygenase/lipoxygenase inhibitor BW 755c potentiated relaxation to bradykinin and had little effect on relaxation in response to arachidonic acid. It is therefore unlikely that lipoxygenase products of arachidonic acid metabolism mediate either bradykinin- or arachidonic acid–induced relaxation of the PCA. The mechanism by which BW 755c potentiated relaxation was not addressed in the present study. The cytochrome P-450 inhibitor clotrimazole partially attenuated arachidonic acid–induced relaxation of PCA rings. It is therefore possible that the relaxation produced by arachidonic acid administration is in part mediated by epoxygenase metabolites, although this question remains to be resolved, because a second inhibitor of cytochrome P-450, proprafen, had little effect on arachidonic acid–induced relaxations. In contrast, bradykinin-induced relaxations were unaffected by either of the cytochrome P-450 inhibitors; therefore, epoxygenase products of arachidonic acid metabolism most likely do not mediate bradykinin-induced relaxation of the porcine coronary artery.

The finding that relaxations in response to arachidonic acid were inhibited by clotrimazole, whereas relaxations in response to bradykinin were not, does not preclude a role for arachidonic acid in mediating bradykinin relaxation. It has been reported that tissues may produce different proportions of eicosanoids depending on the particular agonist used, possibly related to release of arachidonic acid into distinct subcellular sites where it may be metabolized by different enzyme pathways. Thus it is possible that arachidonic acid may be producing relaxation in response to bradykinin through a metabolic pathway that is insensitive to the inhibitors used in the present study. Alternatively, arachidonic acid has been proposed to participate in the cellular signaling process through complex effects such as increasing [Ca²⁺].

In summary, we have demonstrated that arachidonic acid, similar to bradykinin, produces endothelium-dependent relaxation of PCA rings through a mechanism that is sensitive to excess potassium. A role for arachidonic acid in mediating bradykinin relaxation is suggested by the observation that the phospholipase inhibitors quinacrine and BPB attenuated bradykinin- but not arachidonic acid–induced relaxation of PCA rings. In view of the observation that inhibitors of cyclooxygenase-, lipoxygenase-, and cytochrome P-450–mediated metabolism of arachidonic acid did not attenuate bradykinin relaxation, arachidonic acid may produce relaxation through enzymatic conversion that is insensitive to the inhibitors tested or by participating in the cellular signaling process as a second messenger.

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