Improvement by Phosphoramidon of Damaged Endothelial Function in Porcine Coronary Artery

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Background. **The bradykinin (BK)-induced endothelium-dependent relaxation is impaired in the presence of elevated potassium concentration enhancing the vasospastic tendency of large coronary arteries. Inhibition of the angiotensin-converting enzyme responsible for bradykinin degradation was found to enhance the endothelium-dependent relaxation by BK. The aim of the present study was to investigate the effect of phosphoramidon, known to inhibit a BK-metabolizing neutral endopeptidase enzyme, on relaxation of porcine-isolated coronary artery in depolarizing solution.**

Methods. **Endothelium intact porcine coronary artery rings were studied in organ chambers. The rings were isometrically contracted with potassium chloride (30 mmol/L) and the response to BK (1 to 1,000 nmol/L)-induced relaxation was investigated in the presence of nitric oxide synthase inhibitor N$^{\omega}$-nitro-L-arginine (300 μmol/L) alone and in combination with the cyclooxygenase inhibitor indomethacin (10 μmol/L), and that of the inhibitor of calcium-dependent potassium channels tetraethylammonium (7 mmol/L). Under these conditions, phosphoramidon (10 μmol/L), an inhibitor of a neutral endopeptidase enzyme (EC.3.4.24.11.), which is responsible for the degradation of BK, was used to enhance the endothelium-dependent relaxation.**

Results. **Phosphoramidon potentiated the maximum vasorelaxant effect of BK in N$^{\omega}$-nitro-L-arginine (control 26.6% ± 10.86% versus phosphoramidon 49.05% ± 4.52%; n = 6, $p < 0.05$) or in N$^{\omega}$-nitro-L-arginine + indomethacin-pretreated rings (control 20.7% ± 9.92% versus phosphoramidon 42.0% ± 12.26%; n = 5, $p < 0.05$) and this increased vasodilation was not modified by tetraethylammonium.**

Conclusions. **In the present study phosphoramidon potentiated the effect of BK in the absence of nitric oxide and prostaglandins in porcine-isolated coronary artery. This effect did not depend on tetraethylammonium-sensitive potassium channels. Phosphoramidon may be a useful pharmacologic tool for preserving the vasorelaxing capacity of coronary arteries after cardioplegia.**

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Hyperkalaemic solutions are widely used for myocardial protection in cardiac transplantation. For organ perfusions the intact functional endothelium of the arteries is of crucial importance. The vascular endothelium plays an important role in the local regulation of the coronary tone by releasing a number of vasodilating susbstances, such as endothelium-derived nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) [1, 2]. Dysfunction of the endothelium has been shown to enhance the vasospastic tendency in the coronary circulation and increase thrombus formation [3].

It has been reported recently that in porcine coronary artery the endothelium-dependent relaxation mediated by the noncyclooxygenase and nonnitric oxide pathways, that is by EDHF, is impaired in the presence of elevated extracellular potassium concentration [4, 5]. Bradykinin (BK) is known to be an important endogenous regulator of the vascular tone and is able to increase nitric oxide, prostacyclin, and EDHF when the endothelium is intact. The functional role of EDHF could be studied in BK-induced relaxation during which hyperpolarization of the smooth muscle membrane was also detected [6]. The pharmacologic modulation of the degradation of BK by inhibiting the angiotensin-converting enzyme (ACE) was shown to potentiate the coronary dilating effect of BK [7]. The other pathway of BK metabolism [8] involving a neutral endopeptidase enzyme (NEP, EC. 3.4.24.11.) has recently gained interest in the regulation of local vasomotor tone [9]. In that study, phosphoramidon, which has an inhibitory effect on NEP enzyme, increased vascular nitric oxide production suggesting the involvement of endogenous BK in its mechanism of action. At present, however, there is no information about the direct interaction between phosphoramidon and BK as a possible therapeutic intervention for preserving coronary arterial relaxation.

In the present study we investigated the effect of phosphoramidon on the endothelium-dependent relaxation induced by exogenous BK under depolarizing conditions. The response of the porcine-isolated coronary arteries was studied in the absence and presence of

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endothelial inhibitors, indomethacin (INDO), N^{ω}-nitro-L-arginine (L-NA), and that of the calcium-activated potassium channel blocker tetraethylammonium (TEA).

Material and Methods

Coronary arteries were obtained from porcine hearts that were harvested in a local abattoir. After removing the heart it was placed into ice-cold Krebs Henseleit solution (composition in mmol/L: NaCl 120; KCl 4.2; $CaCl_2$ 1.5; $NaHCO_3$ 20; $MgCl_2$ 1.2; KH_2PO_4 1.2; and glucose 11) and transported to the laboratory within 1 hour. Coronary arteries of the circumflex branch were dissected free from the surrounding connective tissue and cut into rings of 5 mm long. Ring segments were mounted on a pair of stainless steel hooks and placed into water-thermostated (at 37°C) organ chambers containing 2 mL of Krebs Henseleit solution. The solution was continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 (pH 7.4). One of the hooks was anchored inside the organ chamber and the other one was connected to a force-displacement transducer (Experimetria, Budapest, Hungary) to measure changes in isometric tension as previously described by Torday and colleagues [10]. The rings were stretched up to 3 g, and equilibrated for 90 minutes. During this period tension was continuously readjusted to the above value of stretch and the medium was changed in every 15 minutes.

Protocols

EFFECT OF ENDOTHELIAL INHIBITORS ON BRADYKININ-INDUCED CORONARY RELAXATION. Endothelium intact coronary artery rings mounted in separate organ chambers were contracted with 30 mmol/L potassium chloride. When the contraction reached a plateau (usually after 10 minutes) BK was administered cumulatively (1 to 1,000 nmol/L) into the organ baths. In other experiments the effect of BK (1 to 1,000 nmol/L) as described above was investigated in the presence of L-NA (300 μmol/L), a nitric oxide synthase inhibitor or INDO (10 μmol/L), a cyclooxygenase inhibitor or TEA (7 mmol/L), a blocker of calcium-activated potassium channels. L-NA, INDO, or TEA were added 30 minutes before the potassium chloride (30 mmol/L)-induced constriction.

EFFECT OF THE NEUTRAL ENDOPEPTIDASE INHIBITOR PHOSPHORAMIDON ON BRADYKININ-INDUCED RELAXATION. In another series of experiments the possible capacity of phosphoramidon to potentiate the BK-induced relaxation was investigated in the presence of L-NA alone, or in combination with INDO and also with TEA. In each experiment two parallel endothelium intact rings were mounted in separate organ baths. Both rings were pretreated with L-NA (300 μmol/L) or INDO (10 μmol/L) + L-NA (300 μmol/L). After 10 minutes phosphoramidon was added in a concentration of 10 μmol/L to one of the rings and the corresponding volume of the phosphoramidon solvent (20 μL in 96% ethanol) to the other one. After an additional 30 minutes rings were contracted with 30 mmol/L potassium chloride and concentration relaxation curves for BK (1 to 1,000 nmol/L) were established. Then at the maximum of BK-induced relaxation TEA (7 mmol/L) was administered to both rings.

Data Analysis

Contractions induced by 30 mmol/L potassium chloride were expressed in grams. The effective concentration of BK, which caused 50% of maximal relaxation was defined as EC_{50}. For the calculation of 50% effective concentration (EC_{50}) values the $(a \times x)/(x + b)$ logistic equation was fitted to the individual dose–response values. From these fitted equations, the mean EC_{50} value $\pm$ standard error of the mean was calculated for each group.

Statistical Analysis

All data are expressed as means $\pm$ standard error of the mean. Statistical significance was tested with Student's two-tailed paired and unpaired t test. Values of p less than 0.05 were considered statistically significant.

Drugs

Drugs used and their sources were as follows: BK-acetate, N^{ω}-nitro-L-arginine, indomethacin, tetraethylammonium and phosphoramidon (Sigma, St. Louis, MO). Bradykinin and tetraethylammonium (dissolved in distilled water), indomethacin, and phosphoramidon (dissolved in 96% ethanol) were stored at 4°C. The solution of L-NA (solvent: distilled water) was held frozen until required.

Results

Characterization of Bradykinin-Induced Relaxation in Porcine Epicardial Coronary Artery

The effect of endothelial inhibitors on BK-induced relaxation is depicted in Figure 1. L-NA partially inhibited the vasorelaxation induced by 1 μmol/L BK, (BK alone: 56.75% $\pm$ 4.81%; versus after L-NA treatment 26.6% $\pm$ 10.86%; $p < 0.05$), whereas INDO caused further decrease of coronary tone (72.15% $\pm$ 2.86%, $p < 0.05$) compared to BK alone. These results support the involvement of NO and point to the role of vasoconstrictor prostaglandins in the effect of BK. TEA reduced the vasodilator response to BK (BK alone: 54.86 $\pm$ 4.61% versus after TEA treatment 42.72 $\pm$ 4.01%, $p < 0.05$). This effect of the potassium channel blocker reveals the role of hyperpolarizing potassium channels in the vasorelaxing mechanism of BK.

Effect of Phosphoramidon on Bradykinin-Induced Relaxation in the Presence of Different Inhibitors of Endothelial Mediators

In this set of experiments the two rings with endothelium were pretreated with L-NA (300 μmol/L) before addition of potassium chloride. Under this condition phosphoramidon did not significantly affect the potassium chloride-induced contraction; control value was 4.13 $\pm$ 0.18 g versus phosphoramidon treatment 4.0 $\pm$ 0.38 g ($n = 6$). In L-NA-treated rings phosphoramidon significantly increased the maximum of BK-induced relaxation from the

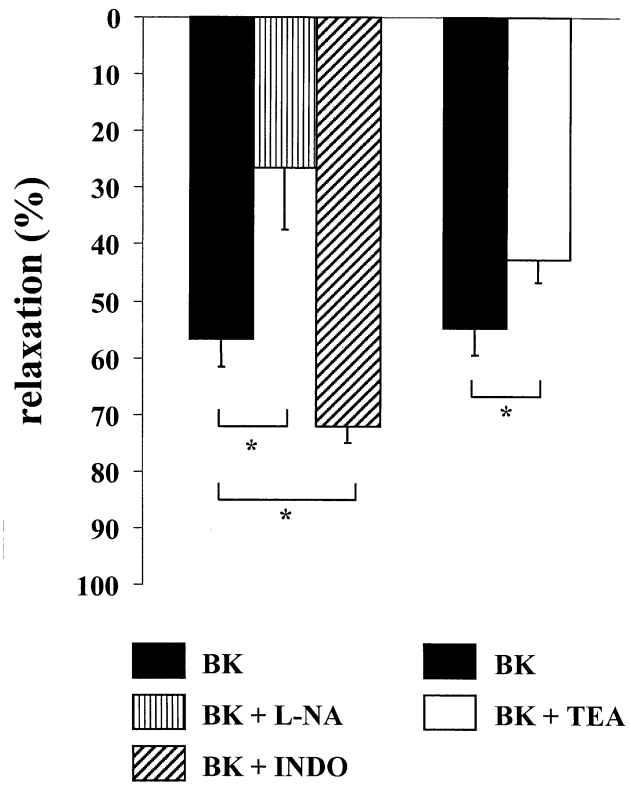

*Fig 1. Vasorelaxing effect of bradykinin (*BK*; 1 μmol/L) in porcine coronary artery in the presence of endothelial inhibitors N^{ω}-nitro-L-arginine (*L-NA*; 300 μmol/L), indomethacine (*INDO*; 10 μmol/L), and tetraethylammonium (*TEA*; 7 mmol/L). Relaxations are expressed as a percentage of the contraction induced by 30 mmol/L potassium chloride and shown as mean ± standard error of the mean (n = 5 to 8). Asterisk denotes significant differences (p < 0.05) between the corresponding groups.*

control value of 26.60% ± 10.86% to 49.05% ± 4.52% (Fig 2A). The difference was significant only above 10 nmol/L BK. Nevertheless, no significant difference was found when the EC_{50} values in the absence and presence of phosphoramidon were compared (−7.22 ± 0.17 log mol/L versus −7.34 ± 0.16 log mol/L; n = 6).

In L-NA- and INDO-pretreated rings phosphoramidon did not affect the maximum potassium chloride-induced contraction (control 2.49 ± 0.84 g versus after phosphoramidon treatment 1.90 ± 0.43 g; n = 5).

Similar to L-NA-treated rings, phosphoramidon significantly enhanced the vasorelaxant effect of BK in the presence of INDO and L-NA (Fig 2B). The maximal relaxation was 20.70% ± 9.92% under control conditions and 42.00% ± 12.26% in the presence of phosphoramidon treatment. This enhancement of vasorelaxing effect was not followed by a significant shift in EC_{50} values (−7.74 ± 0.20 log mol/L versus −7.76 ± 0.16 log mol/L; n = 5).

Phosphoramidon did not change the effect of TEA on BK-induced relaxation in the presence of L-NA and INDO (in the absence of phosphoramidon 11.60% ± 16.10% versus in the presence of phosphoramidon 1.60% ± 8.50%; n = 5).

Comment

In the present study, we have demonstrated that in hyperkalemic solution phosphoramidon enhanced the endothelium-dependent relaxation induced by exogenous BK.

In the first part of experiments we characterized the feature of BK-induced relaxation in porcine isolated coronary preparations. In the presence of functional endothelium the modulation of the effect of BK on coronary tone was evaluated by combining BK with known endothelial inhibitors: INDO, L-NA, and TEA. TEA is known to inhibit the relaxing and hyperpolarizing effects of EDHF in large epicardial arteries of the porcine heart [11]. By using these inhibitors, we found that two endothelium-derived mediators were responsible for the relaxation evoked by BK. These were nitric oxide and a TEA inhibitable EDHF. This finding is in agreement with previous observations in the coronary artery of the same species [11, 12]. INDO enhanced the relaxation by 1 μmol/L BK indicating the release of functionally effective vasoconstrictor prostaglandins rather than prostacyclin under our experimental conditions. However, this significant increase of BK-induced relaxing effect by INDO may not reflect the vasoactive prostaglandin balance because INDO by itself significantly decreased the potassium chloride-induced contraction (data not shown). It is generally accepted that a lower precontraction force facilitates relaxation. In the presence of L-NA, the maximum relaxation by 1 μmol/L BK was decreased by 66% compared to the effect of BK alone and by 71% compared to the INDO plus L-NA-treated blood vessels. This suggests that a considerable part of the vasodilating action of BK involves EDNO, and prostaglandins do not play a considerable role in the vasoactivity of BK on porcine coronary artery. In the presence of TEA the relaxation induced by BK was slightly (by 22%), but significantly decreased. This finding provides evidence for the role of EDHF in BK-induced coronary dilation.

Pharmacologic modulation of BK-induced endothelium-dependent relaxation could be achieved by inhibition of the angiotensin-converting enzyme and the therapeutic relevance of this type of interaction is well established in cardiovascular disorders. Besides angiotensin-converting enzyme, a specific neutral endopeptidase (NEP, EC. 3.4.24.11.) also participates in the breakdown of BK; however, the significance of inhibition of this NEP enzyme is not exactly known. Llorenz-Cortes and colleagues [13] identified and characterized the NEP in endothelial cells of venous and arterial vessels and demonstrated that 50% of BK hydrolysis in vascular tissue was due to NEP activity. Studying the role of NEP in the degradation of BK in cultured human endothelial cells Graf and associates [14] found that the NEP inhibitor phosphoramidon significantly diminished the breakdown of BK. We cannot exclude the possibility that another mechanism of phosphoramidon, the inhibition of endothelin-converting enzyme [15], also participates in the enhancement of BK-induced relaxation in our current study. The decrease of the activity of this endothelial

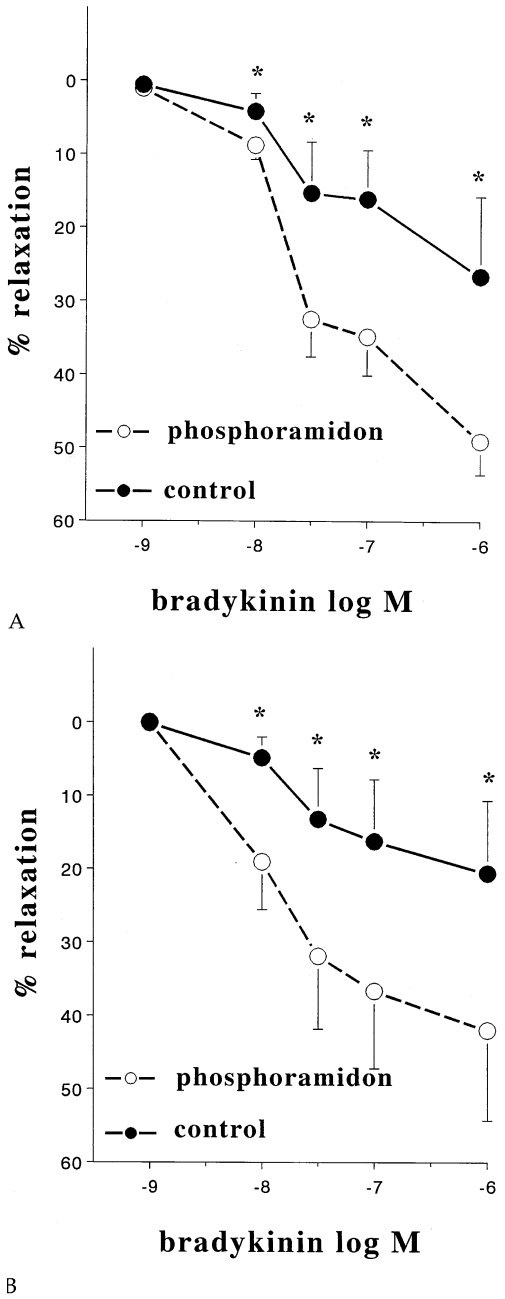

Fig 2. Effect of phosphoramidon (10 μmol/L) on bradykinin-induced relaxation in the porcine coronary artery rings pretreated with N^{ω}-nitro-L-arginine (300 μmol/L) (A) or with indomethacine (10 μmol/L) + N^{ω}-nitro-L- arginine (300 μmol/L) (B). The rings were contracted with potassium chloride (30 mmol/L) and bradykinin (1 to 1,000 nmol/L) was applied in a cumulative manner. The relaxation of the tissue by bradykinin was expressed as a percentage of the contraction induced by potassium chloride. Values are expressed as means ± standard error of the mean (● = absence of phosphoramidon; ○ = presence of phosphoramidon (10 μmol/L) (n = 5 to 6). Asterisk denotes significant differences ($p < 0.05$) between the corresponding values of the groups with and without phosphoramidon pretreatment.

enzyme may suppress the production of vasoconstrictor endothelin enabling the vasodilator mediators to produce more relaxation. The latter mechanism of phosphoramidon appears to be operative in systemic circulation [15] but not in coronary tree. Phosphoramidon enhanced coronary flow and conductance of the rat heart exclusively through the BK-2 receptor [16] suggesting that the primary role of the drug is the inhibition of the metabolism of BK without a considerable effect on the production of endothelin in the coronary circulation.

The present experimental arrangement can be regarded as the simulation of functional endothelial damage in that a clear improvement of coronary function by phosphoramidon could be demonstrated. In the absence of EDNO, that is, in the experiments with L-NA, phosphoramidon potentiated the effect of BK virtually to the same extent as in the case of the suppression of both EDNO and prostaglandins (compare Fig 2A with Fig 2B). This finding supports the abovementioned assumption that vasoactive prostaglandins do not have an important role in the regulation of coronary tone of the porcine heart under our conditions. The same conclusion was drawn on the basis of the results of experiments performed in hyperkalemic nutrient solution with the other endothelial stimulator, substance-P, on porcine isolated coronary preparations [5].

In the absence of EDNO, the BK-induced relaxation represents EDHF, the effect of which could be increased by phosphoramidon. The nature of this TEA-sensitive EDHF has not yet been determined. EDHF is an activator of the calcium-dependent potassium channels, which hyperpolarizes the membrane of the smooth muscle cells and causes relaxation [12, 17]. The limitation of our present experiment is that we cannot exclude a possible direct interaction between phosphoramidon and EDHF. We postulate that the concentration of BK in coronary tissue is enhanced as a consequence of the NEP inhibitory effect of phosphoramidon because the drug was also able to increase the production of EDNO, the other mediator of BK, in dog coronary homogenates [9].

Pharmacologic improvement of the relaxing capacity of the coronary arteries after cardioplegia (ie, cardiac transplantation or operation) is of crucial importance. Phosphoramidon is a candidate for the enhancement of endothelium-dependent relaxations in depolarizing conditions and may serve as a useful pharmacologic tool by enhancing coronary vasorelaxation after cardioplegia.

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INVITED COMMENTARY

Depolarizing cardioplegia has been a standard and effective method to arrest and protect the heart during open-heart surgery. With increasing knowledge about the vascular endothelial function, particularly the discovery of not only nitric oxide (NO), but also other pathways such as so-called endothelium-derived hyperpolarizing factor (EDHF) has made the understanding on the physiology and pathophysiology of coronary circulation during cardioplegic arrest more complete. We have, in the recent years, reported the adverse effect of depolarizing cardioplegia on the EDHF-mediated pathway [1, 2]. Although the clinical role on this is still unclear because the strong effect of NO may well cover the effect of other factors, at least in critically ill patients, the complete protection of all functions of the coronary endothelium is beneficial to the perfusion of the myocardium and the recovery of the heart. One of the ways to protect the coronary endothelium is to use hyperpolarizing, rather than depolarizing cardioplegia [3]. The present study reports another possible method to protect the non-NO pathway, ie, using angiotensin converting enzyme (ACE) inhibitors to reduce the degradation of bradykinin and therefore to enhance the bradykinin-mediated endothelium-dependent relaxation. Although this is an interesting thinking and the experimental data are reliable, it is unknown what role bradykinin plays in the cardiac arrest period and perioperatively. In the laboratory setting, bradykinin is used as an "index" of endothelial function rather than as an important biological messenger, per se. Therefore, future studies should be designed to investigate whether the ACE inhibitor may affect the endothelial function in other aspects. In view of protection of the endothelial function, it will be a long way from the present study to the possible clinical use.

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