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Effects of Rp-8-Br-cGMPS, a selective inhibitor of activation of cyclic GMP-dependent protein kinase by cyclic GMP, on relaxation of the rat aortic smooth muscle induced by nitroglycerin and nitroprusside.

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To examine the importance of activation of cyclic GMP-dependent protein kinase (protein kinase G) by cyclic GMP in relaxation of vascular smooth muscle by nitroglycerin and nitroprusside, effects of Rp-8-Br-guanosine 3',5'-cyclic monophosphorothioate (Rp-8-Br-cGMPS), a selective, membrane permeable inhibitor of activation of protein kinase G by cyclic GMP, were studied using the isolated ring preparations of the rat aorta. Relaxation of high K^+-contracted preparations by nitroglycerin and nitroprusside which has been repeatedly shown to occur in association with lowering of Ca^{2+} sensitivity of contractile protein was effectively counteracted by Rp-8-Br-cGMPS in harmony with our recent findings on the intimate connection of Ca^{2+} sensitivity lowering with activation of protein kinase G[1], while relaxation of phenylephrine-contracted preparations generally considered to proceed in connection with lowering of intracellular concentrations of Ca^{2+} was not.

Key words: nitroglycerin, nitroprusside, cyclic GMP-dependent protein kinase, Rp-8-Br-cGMPS, rat aorta

I Introduction

Relaxation of vascular smooth muscles by nitroglycerin and nitroprusside is presumed to occur through activation of cyclic GMP-dependent protein kinase (protein kinase G) by a second messenger, cyclic GMP, and consequent phosphorylation of a target protein or proteins of this enzyme[2,3].

To clarify whether activation of protein kinase G is indeed involved in relaxation of vascular smooth muscles produced by these compounds, experiments were performed using Rp-8-Br-guanosine-3', 5'-cyclic monophosphorothioate (Rp-8-Br-cGMPS), a membrane-permeable derivative of Rp-guanosine-3', 5'-cyclic monophosphorothioate (Rp-cGMPS) which was shown by Butt el al. (1994)[4] to be a selective inhibitor of activation of protein kinase G by cyclic GMP. That Rp-8-Br-cGMPS is a selective inhibitor of activation of protein kinase G by cyclic GMP has been demonstrated in a previous paper[5].

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II Methods

Experiments were performed with isolated smooth muscle preparation of the rat aorta. Male Wistar rats (300-400g) were sacrificed by exsanguination under light anesthesia with ether. The thoracic arota was rapidly excised, placed in a chamber filled with an ice-cold, modified Krebs-Henseleit (K-H) solution and dissected free of fat and connective tissues.

Endothelial cell layer was removed by gentle rubbing of the luminal surface with a stainless-steel rod to eliminate the effects of drugs on endothelial cells, and the endothelium-denuded aorta was cut into rings about 2 mm long. The ring preparation was suspended between two stainless steel wire hooks in a 10 ml organ bath containing K-H solution of the following composition: NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; glucose, 10.0 (mM). The solution was aerated with 95 % O_2 + 5% CO_2 and kept at 37℃. Isometric tension developed by the preparations was measured with a force-displacement transducer (T7-30-240, Orientec Japan, Tokyo,

Japan) connected to a carrier amplifier (6M81, NEC San-ei, Tokyo, Japan) and recorded on a potentiometric recorder (Servocorder SR 6211, Graphtec, Tokyo, Japan).

After an equilibration period of 60-90 min under an optimal resting tension of 2 g with every 15 minutes changes of the solution, the preparations were contracted with 0.1 μM of phenylephrine. After attainment of a steady tension, 1 μM of acetylcholine was administered to test the completeness of removal of endothelium. No preparations responded to acetylcholine.

After several times washings and recovery of a steady resting tension preparations were treated with 30 μM of Rp-8-Br-cGMPS for 1 or 5 hr. Then, the preparations were contracted either by addition of 0.1 μM of phenylephrine or by replacement of the bathing solution by a high K^+ K-H solution (all NaCl was substituted with KCl in this solution) containing 30 μM of Rp-8-Br-cGMPS. After attainment of a stable contractile tension (ca. 40 min later), nitroglycerin and nitroprusside were administered cumulatively.

Results were expressed as mean ± SEM. Statistical significance of the data was evaluated using Student's t test. When probability was less than 0.05, the difference was considered significant.

Drugs used were: Rp-8-Br-cGMPS (Biolg, Bremen, FRG), nitroglycerin (Nippon Kayaku, Tokyo, Japan) and phenylephrine (Kowa, Nagoya, Japan). Sodium nitroprusside, acetylcholine and other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Animals used received humane care in compliance with the "Principles of Laboratory Animalcare" (NIH publication No.85-23, revised in 1985) and the "Guide for the Animal Experimentation" formulated in this University.

Ⅲ Results

Fig. 1 depicts the effects of Rp-8-Br-cGMPS on the relaxation induced by nitroglycerin and nitroprusside in smooth muscle preparations of the rat aorta contracted with phenylephrine or high K^+ K-H solution. Incubation of the preparations with Rp-8-Br-cGMPS was for 1 hour.

In phenylephrine-contracted preparations, Rp-8-Br-cGMPS induced a small, but significant rightward shift of the concentration-response curve for relaxation by nitroglycerin, but was without significant effects on the concentration-response curve for relaxation by nitroprusside. Prolongation of incubation with Rp-8-Br-cGMPS to 5 hours resulted in no augmentation of the shift to the right of the concentration relaxation curves (data not shown). Neither did insertion of the period of incubation with Rp-8-Br-cGMPS of 1 hr in the presence of high K^+ between the periods of incubation with the compound in normal K-H solution of 3 and 1 hrs produce any augmentation of the right-ward shift of the concentration response curve for relaxation by these two compounds.

Ⅳ Discussion

In the present study higher concentrations of nitroglycerin were necessary to induce relaxation in high K^+-contracted preparations and the relaxant effects were weaker as compared with those in preparations contracted with phenylephrine. This was also the case with nitroprusside; the concentrations necessary to induce relaxation were about 100 times greater as compared with those necessary to induce relaxation in phenylephrine-contracted preparations. These findings indicate that a mechanism or mechanisms through which lower concentrations of these two compounds produce relaxation in normal K-H solution was not operative in preparations contracted by high K^+ K-H solution. As such a mechanism activation of large-conductance, Ca^{2+}-activated K^+-channel (BK channel) as demonstrated by Bolotina et al. (1994)[6] with NO using cell-free membrane patches from rabbit aortic smooth muscle cells is conceivable. BK channel was demonstrated to exist in the rat aortic smooth muscle cells[7].

In recent years measurement of intracellular concentrations of Ca^{2+} with fluorescent dyes has become very popular and it has repeatedly been demonstrated that the intracellular concentration of Ca^{2+} did not decrease when smooth muscle preparations precontracted with high K^+ medium were relaxed by nitroglycerin or nitroprusside, while there

was a definite decrease in intracellular concentrations of Ca^{2+} when the preparations precontracted with agonists such as phenylephrine were relaxed by these two compounds[8-11]. Thus, it has become generally admitted that relaxation of high K^+-contracted smooth muscle by nitroglycerin and nitroprusside is due to lowering of Ca^{2+} sensitivity of contractile proteins. Using skinned vascular smooth muscle preparations, Nishimura and van Breemen (1989)[12] demonstrated that cyclic GMP could produce a reduction of Ca^{2+} sensitivity of contractile proteins. We have succeeded to demonstrate the involvement of activation of protein kinase G in the observed reduction of Ca^{2+} sensitivity by cyclic GMP[1]; in skinned smooth muscle preparations of the rat mesenteric artery, 8-Br-cGMPS produced a complete reversal of the shift to the right of the concentration-contraction curve for Ca^{2+} induced by 8-Br-cGMP, at a concentration at which it did not produce any effect on the concentration-contraction curve for Ca^{2+}, indicating that activation of protein kinase G represented the main mechanism of lowering of Ca^{2+} sensitivity of the contractile proteins induced by cGMP.

The findings of the present study that a selective inhibitor of activation of protein kinase G, Rp-8-Br-cGMPS, produced a definite right-ward shift of the concentration-response curve for relaxations produced by nitroglycerin and nitroprusside in high K^+-contracted preparations, while the shift was minimal or even absent in preparations contracted with phenylephrine are in harmony with the idea that lowering of Ca^{2+} sensivitiy was the main mechanism for relaxation of high K^+-contracted vascular smooth muscles by nitroglycerin and nitroprusside and that the lowering of Ca^{2+} sensivity was the result of activation of protein kinase G by cGMP.

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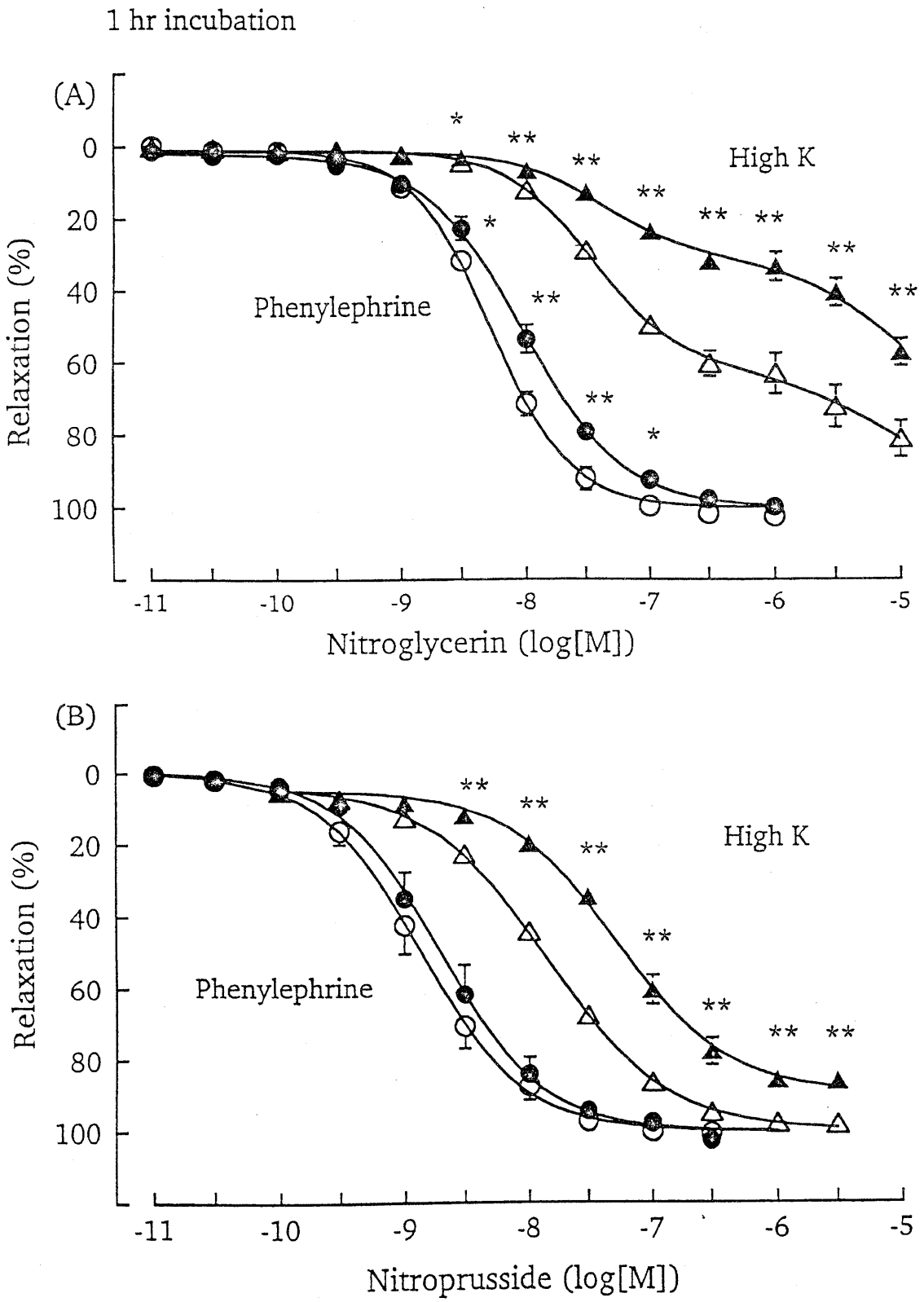

Fig. 1 Effects of 1 hr incubation with Rp-8-Br-cGMPS (30 μM) on the relaxation by nitroglycerin and nitroprusside of rat aortic smooth muscle preparations contracted with phenylephrine or high K^+-Krebs Henseleit solution (high K^+). Each point represents mean ±SEM. Open symbols: control rings, closed symbols: rings treated with Rp-8-Br-cGMPS. * and ** indicate significant differences from control rings. $P<0.05$ and $P<0.01$, respectively.

cGMP依存性プロテインキナーゼのcyclic GMPによる活性化を選択的に抑制する物質であるRp-8-Br-guanosine 3',5'-cyclic monophosphorothioate(Rp-8-Br-cGMPS)の、ニトログリセリン、ニトロプルシッドによるラット大動脈平滑筋弛緩に対する作用

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血管平滑筋のニトログリセリン、ニトロプルシッドによる弛緩におけるcyclic GMP依存性プロテインキナーゼ（プロテインキナーゼG）の重要性を確かめる為、ラット大動脈の摘出リング標本を用い cyclic GMPによるプロテインキナーゼGの活性化を選択的に抑制する、膜透過性の抑制薬であるRp-8-Br-guanosine 3',5'-cyclic monophosphorothioate（Rp-8-Br-cGMPS）の作用について検討した。収縮蛋白のCa^{2+}感受性低下と関連して起こる事が繰り返し示されている、高濃度K^{+}で収縮させた標本のニトログリセリン、ニトロプルシッドによる弛緩は、Rp-8-Br-cGMPSによって著明に抑制された。これはCa^{2+}感受性の低下は、プロテインキナーゼGの活性化と密接に結びついているという我々の最近の所見（Kawada, Toyosato, Omedul Islam, Yoshida and Imai, 1997）と一致している。これに反し、細胞内Ca^{2+}濃度の低下と結びついていると一般的に考えられている、フェニレフリン収縮標本のこれら2つの物質による弛緩は、Rp-8-Br-cGMPSによって抑制されなかった。

キーワード：　ニトログリセリン、ニトロプルシッド、cyclic-GMP依存性プロテインキナーゼ、Rp-8-Br-cGMPS、ラット大動脈

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