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Inhibition of protein kinases A and G by hydralazine but not KRN2391 *in vitro*.

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AIM: To examine possible direct effects of the vasodilators hydralazine and KRN2391 on the activities of protein kinase A (PKA), protein kinase G (PKG), and protein kinase C (PKC). **METHODS**: PKA, PKG and PKC were extracted from bovine lung, heart and rat brain and purified to homogeneity by chromatography. The effects of different reagents on these protein kinase activities were determined *in vitro*. **RESULTS**: Hydralazine (0.03—10 mmol·L^{-1}) inhibited the activities of both PKA and PKG with IC_{50} of 1.2 and 2.5 mmol·L^{-1}, respectively, but had little effect on PKC. KRN2391 (1—1000 μmol·L^{-1}) had no effects on PKA, PKG, or PKC. Using H-89 and GF109203X, inhibitors relatively selective to PKA and PKC respectively, as controls, we obtained inhibitions on PKG, PKA, and PKC similar to literature. However, a novel PKG inhibitor KT5823 failed to inhibit PKG or PKA. **CONCLUSION**: Hydralazine alters the activity of PKG and PKA, which may have implications for the vasodilator activity.

KEY WORDS hydralazine; KRN2391; cyclic AMP-dependent protein kinases; protein kinases; protein kinase C; cyclic GMP-dependent protein kinases

Hydralazine is an arterial dilator used for many years to treat congestive heart failure. KRN2391, *N*-cyano-*N*′-(2-nitroxyethyl)-3-pyridinecarboximidamide monomethanesulphonate, is a novel vasodilator possessing a dual mechanism of action as a nitrate and as a K^+ channel opener[1], which increases coronary, renal, and mesenteric blood flow[2]. It remains unknown how these compounds induce relaxation. Protein kinases enzymes play a pivotol regulatory role in the mechanisms underlying vasoconstriction or vasodilation. In particular, activation of cAMP- and cGMP-dependent protein kinases (PKA and PKG) in smooth muscle cells induces relaxation. There are also a wide variety of active compounds towards protein kinase enzymes[3-6]. Therefore it may be possible that pharmacological modulation of the activities of these enzymes could modulate vasodilation. In this study, the direct effects of hydralazine and KRN2391 on the *in vitro* activities of PKA, PKG, and PKC were explored to examine the possible mechanisms underlying their biological effects. A widely used PKA inhibitor H-8 (*N*-[2-((3-(4-Bromophenyl-2-propenyl)amino) ethyl]-5-isoquinoline sulfonamide), and a PKC inhibitor GF 109203X (3-[1-(3-dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl)-maleimide) as well as a novel PKG inhibitor KT5823, an indole carbozole, were also used in this study as controls[8,9].

MATERIALS AND METHODS

Protein kinase G was extracted from bovine lung and purified to homogeneity using DEAE cellulose and cAMP-affinity columns[3] with an additional step of Mono-Q chromatography[4] and was stored at −70 ℃ in 10 % glycerol with 0.05 % Tween 80 until use.

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The catalytic subunit of bovine heart PKA was purified[5] except that the hydroxylapatite column was replaced with S-Sepharose chromatography[4]. Rat brain PKC was purified from snap frozen tissue[4]. Briefly, PKC was translocated to the membrane by homogenization with calcium and was then extracted with egtazic acid 5 mmol · L^{-1} and edetic acid 2 mmol · L^{-1}. The extracted PKC was then applied to a high performance Q-Sepharose ion-exchange column and chromatographed with a linear gradient of NaCl 0–0.3 mol · L^{-1} in buffer A (Tris/HCl 20 mmol · L^{-1}, edetic acid 1 mmol · L^{-1}, dithiothreitol 1 mmol · L^{-1}, pH 7.4). PKC peak activity was pooled, diluted with buffer A, reapplied to the same column and eluted under the same conditions except in the presence of ATP 3 mmol · L^{-1} plus Mg^{2+} 3 mmol · L^{-1}, which altered PKC such that it eluted earlier. Finally, the sample was brought to 1 mol · L^{-1} with NaCl and applied to a phenyl-Sepherose column which was washed with NaCl 0.5 mol · L^{-1} and batch eluted in 2-mL aliquots of buffer A without salt. The homogeneous PKC was pooled and adjusted to 10 % glycerol and 0.05 % Triton X-100 and stored at −80 ℃.

The assay for protein kinase activity was carried out in Tris/Mg/egtazic acid/Tween 80 buffer (Tris 30 mmol · L^{-1} pH 7.4, $MgSO_4$ 10 mmol · L^{-1}, egtazic acid 1 mmol · L^{-1}, 0.05 % Tween 80) for PKA and PKG or in Tris/Mg/egtazic acid/calcium buffer (the same as above plus $CaCl_2$ 1.2 mmol · L^{-1}) for PKC and at 30 ℃ in the presence of ATP 200 μmol · L^{-1} [γ-^{32}P]ATP (5.5 × 10^{-4} cpm · mol^{-1}) (New England Nucleus, USA), with the synthetic peptide phospholamban 8–22 (PL8–22, 0.1 g · L^{-1}, synthesized by Macromolecular Resources, USA) as the substrate for PKA and PKG, and glycogen synthase 1–12 (GS1–12, 0.1 g · L^{-1}, synthesized by the Peptide Synthesis Group of Louisianna State University, USA) as the substrate for PKC[6]. cGMP (10 μmol · L^{-1}, Sigma), phosphatidylserine/1,2-diolein (10 mg · L^{-1} and 1 mg · L^{-1}, Sigma) were included in appropriate reaction mixtures to activate PKG and PKC respectively. Assays were linear with time and enzyme concentration and used < 30 % of the substrate peptide[7]. Hydralazine (Ciba-Geigy, Australia) and KRN2391 (a generous gift from Kirin Brewery Co, Gunma, Japan) were dissolved in deionized water. H-89, GF 109203X, and PKI 5823 (all Calbiochem) were dissolved in Me_2SO to 1 g · L^{-1} and further diluted with deionized water. Four μL of the 5 testing drugs were added to the reaction mixture with the final concentrations shown in the Fig 1.

The purified protein kinases (12 μL) were added to the reaction mixture with a total volume of 40 μL to initiate the phosphorylation and the reaction was stopped with 8 μL of H_3PO_4 375 mmol · L^{-1} after 10 min. The reaction mixture (30 μL) was spotted onto P81 cation exchange paper (Whatman) and washed 3 times with H_3PO_4 75 mmol · L^{-1} to remove free [γ-^{32}P]ATP. The paper was dried and counted in a scintillation vial with 10 mL of organic scintillant (Beckman, Ready-Organic). The protein kinase activities were measured as nmol · min^{-1}/mg protein after substracting the basal activity. Basal activity for each protein kinase was obtained by determining activity without any activators and in the presence of the following conditions: PKI (1 mg · L^{-1}) (a gift from Dr J Haycock, USA) for PKA, H-8 (25 μmol · L^{-1}) for PKG; Ca^{2+} (200 μmol · L^{-1}) for PKC. Percent activity relative to vehicle-treated controls was used to analyze differences. Unpaired *t*-test was used to analyze the differences between vehicle-treated controls and drug treated groups.

RESULTS

Hydralazine (0.03–10 mmol · L^{-1}) inhibited both PKA and PKG activities *in vitro* in a concentration dependent manner with IC_{50} of 1.2 and 2.5 mmol · L^{-1}, respectively (Fig 1). In the range of 0.03 – 1 mmol · L^{-1}, hydralazine slightly increased the activity of PKC, but partly inhibited the activity of PKC at 10 mmol · L^{-1}. KRN2391 (1 – 1000 μmol · L^{-1}) had no direct effect on the activity of PKC and little effect on PKA and PKG. It only slightly inhibited the activities of PKG and PKA at concentrations >1000 and >100 μmol · L^{-1}, respectively (Fig 1).

GF109203X showed a more potent inhibition of PKC than PKG and PKA with IC_{50} of 1.80 nmol · L^{-1}, 0.20, and 0.56 μmol · L^{-1}, respectively (Fig 1). H-89 showed a stronger inhibition on PKA than on PKG and PKC with

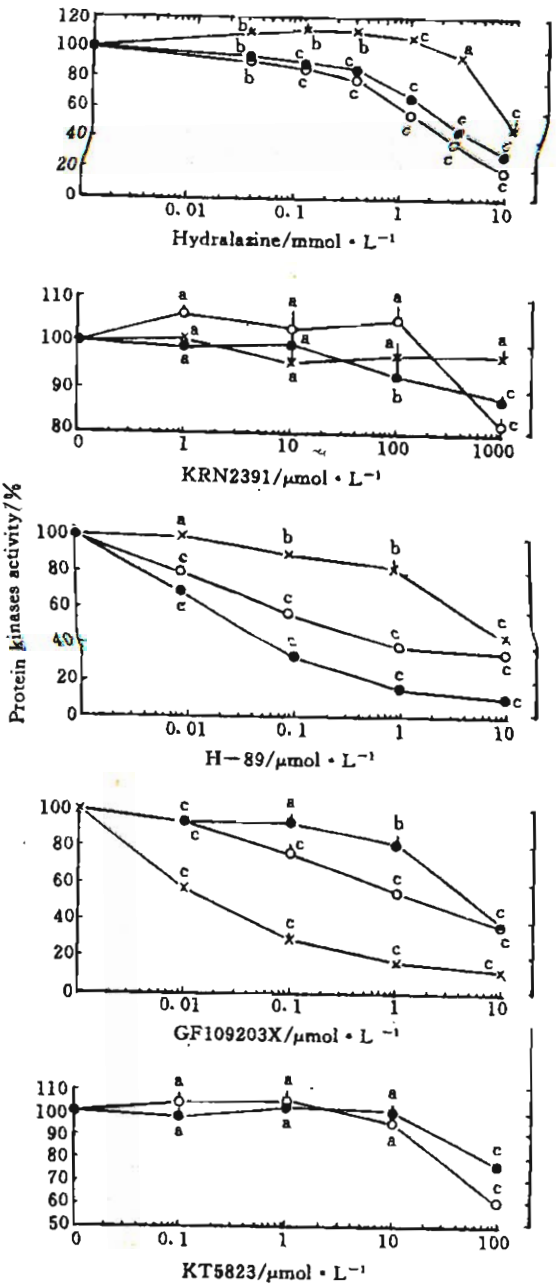

Fig 1. Effects of hydralazine, KRN2391, H-89, GF109203X, and KT5823 on activities of PKG (○), PKA (●), and PKC (×) *in vitro*. $n=3$. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* vehicle control.

IC_{50} values of 0.03, 0.22, and 6.90 μmol·L^{-1} respectively. KT5823 (0.1—10 μmol·L^{-1}) did not inhibit PKG and PKA; and only inhibited PKA and PKG by 22 % and 40 % respectively at 100 μmol·L^{-1} (Fig 1).

The absolute values of PKA, PKG and PKC activities for vehicle treated controls are 1.43, 0.47, and 0.57 μmol·mL^{-1}·min^{-1}, respectively for hydralazine study; 7.31, 2.04, and 0.35 μmol·mL^{-1}·min^{-1}, respectively for KRN2391; 7.73, 2.09, and 0.40 μmol·mL^{-1}·min^{-1}, respectively for H-89; 7.69, 1.96, and 0.35 μmol·mL^{-1}·min^{-1}, respectively for GF109203X; For KT5823 study, the absolute values of PKA and PKG for vehicle treated controls are 8.39 and 2.08 μmol·mL^{-1}·min^{-1}.

DISCUSSION

In this study we have compared the effects of two vasodilators on the activities of three purified protein kinase enzymes, PKG, PKA and PKC with the effects of known, potent inhibitors of these enzymes. Both vasodilators failed to significantly activate the protein kinases, and hydralazine proved to be an inhibitor of PKG and PKA and a weaker inhibitor of PKC. The known inhibitors produced the expected potencies and protein kinases specificity.

The known inhibitor of PKC, GF 109203X, potently inhibited the enzyme over 1000 times more selectively than PKG or PKA. These results were essentially the same as previously reported for PKC and PKA[3]. Similarly the known inhibitor of PKA, H-89, potently inhibited PKA 7 or 200 times more selectively than PKG or PKC respectively. These values were also similar to those reported previously[8]. A novel inhibitor, KT5823, with selectivity for PKG has recently been reported[8], although another group report the inability of this compound to inhibit PKG[10]. We also found that KT5823 failed to inhibit PKG or PKA in the range 0.01 to 10 μmol

·L^{-1}, but produced weak, non-selective inhibition at 100 μmol·L^{-1}. This suggests that this compound may not be an effective or selective inhibitor of PKG.

Hydralazine has been used for many years as an arterial dilator, but its mechanism of action is unknown. In this study we found that hydralazine at concentrations producing vasodilation *in vitro*[10,12] inhibited PKA and PKG. The compound also slightly elevated PKC activity at this concentration, but inhibited PKC at higher concentrations. Although the effect of the compound on these enzymes is not as predicted, the potency of the inhibitory actions on PKA and PKG directly relate to its potency in intact tissue, raising the possibility that the effect of hydralazine on PKA and PKG could relate to its action in vasodilation. However, it is not clear how inhibition rather than activation of these enzymes could contribute to vasodilation. Yen *et al* also failed to demonstrate any accumulating effect of hydralazine on cGMP level in the aortae[10]. although others showed that hydralazine released nitric oxide which then activated soluble guanylate cyclase and elevated cGMP to produce vasodilation[12].

KRN2391 is a novel vasodilator possessing a dual mechanism of action as a nitrate and as a K^+ channel opener[1]. In this study, we found that KRN2391 had hardly any direct effects on PKA, PKC and PKG. It only slightly inhibited the activities of PKG and PKA at concentrations much higher than required for its vasodilating effect[2]. Our findings support that the site of action of KRN2391 at a point prior to PKG activation by cGMP.

In conclusion, hydralazine is therefore a novel inhibitor of PKG and PKA, with 2-fold selectivity towards PKG. Although the potency of hydralazine towards protein kinases is low, the possibility remains that modulation of protein kinases in vascular smooth muscle may contribute to the vasodilation produced by this compound.

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肼酞嗪对蛋白激酶 A 和蛋白激酶 G 的体外抑制作用

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目的：研究血管扩张药肼酞嗪和 KRN2391 对蛋白激酶的直接作用以探讨其作用机制. **方法**：采用层析纯化的蛋白激酶进行体外活性测定. **结果**：肼酞嗪可以抑制 PKA 和 PKG 的活性(0.01—10 mmol·L^{-1})，其 IC_{50}分别为1.2和2.5 mmol·L^{-1}，而对 PKC 作用很小. KRN2391 (1—1000 μmol·L^{-1})对 PKA, PKG 和 PKC 活性均无明显影响. **结论**：肼酞嗪对 PKA 和 PKG 的直接作用可能是其扩张血管的机制之一.

关键词 肼肽嗪; KRN2391; cAMP 依赖性蛋白激酶类; 蛋白激酶类; 蛋白激酶 C; cGMP 依赖性蛋白激酶类

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Evoked tensions in rabbit aorta by emptying intracellular Ca^{2+} stores with cyclopiazonic acid, thapsigargin, and ryanodine

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AIM: To study the increase of plasma membrane Ca^{2+} permeability in response to depletion of intracellular Ca^{2+} stores. **METHODS**: In Ca^{2+}-free medium, 2 selective inhibitors of sarcoplasmic reticulum (SR) Ca^{2+} pump ATPase, cyclopiazonic acid (CPA) and thapsigargin (Tha), and an activator of Ca^{2+}-induced Ca^{2+} release channel (CICR), ryanodine (Rya), depleted intracellular Ca^{2+} stores sensitive to both caffeine and phenylephrine in rabbit aortic rings and caused sustained tensions when Ca^{2+} reintroduction. These tensons were taken as the increase of plasma Ca^{2+} permeability by depletion of intracellular Ca^{2+} stores. **RESULTS**: The extracellular Ca^{2+}-dependent tensions caused by Tha and Rya 3 μmol·L^{-1} and CPA 30 μmol·L^{-1} were 0.94, 1.1, and 0.14 g, respectively, and the tension caused by Rya was not inhibited by CPA. **CONCLUSION**: (a) Besides the depletion of intracellular Ca^{2+} stores an activated state of Ca^{2+} release channels in SR may also mediate the activation of Ca^{2+} influx from plasma membrane in rabbit aorta. (b) Rya needs caffeine to fully open CICR channel in SR.

KEY WORDS cyclopiazonic acid; thapsigargin; ryanodine; thoracic aorta; caffeine; phenylephrine

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Sarcoplasmic reticulum (SR) of vascular smooth muscle plays a pivotal role in the maintenance of intracellular Ca^{2+} concentra