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添付資料：研究報告書

受給者氏名：遠藤 政夫 ㊞

所属機関名：山形大学

所属部署：医学部 職名：教授

〒990-9585

所在地：山形市飯田西 2-2-2

電話：023-628-5234 内線：

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心室筋細胞エンドセリン受容体を介する情報伝達機構の解明

3．成果の概要（100字程度）

哺乳類（イヌおよびウサギ）心室筋におけるエンドセリン（ET）とET-1とノルエピネフリンのフロストークによる収縮性およびCa^{2+}シグナルの調節に関与する細胞内シグナル伝達過程の解析について5編の論文を完成し、イギリス(1)、ヨーロッパ(2)、日本(2)の薬理学の英文専門誌に受理され全てすでに出版または印刷中の状態にあり成果は充分にあった。

4．被招聘者

氏名：楚 立 職名：教授

所属機関：中国 河北医科大学 部署：薬理学

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心室筋細胞エンドセリン受容体を介する情報伝達機構の解明

研究者氏名　楚　立
中国所属機関　中国河北医科大学薬理学
日本研究機関　日本山形大学医学部循環薬理学
指導責任者　教授　遠藤　政夫
共同研究者名　石井　邦明，　蓬田　伸一，
西丸　和秀，　野呂田　郁夫

要　旨

Endothelin-1 (ET-1) induces a positive inotropic effect (PIE) due to a combination of an increase in intracellular Ca^{2+} transients (CaT) and myofilament Ca^{2+} sensitivity in rabbit ventricular myocardium, but it alone does not elicit significant inotropic effect in dog ventricular myocardium. However, in the presence of norepinephrine (NE), it induces a PIE and/or negative inotropic effect (NIE) depending on the concentrations of NE coexisting with ET-1. The roles of kinases such as protein tyrosine kinase (PTK), Rho kinase (ROK) and myosin light chain kinase (MLCK) in the regulation of cardiac contractility and Ca^{2+} signaling remains unclear. The present studies were undertaken to examine the effects of PTK inhibitor genistein, ROK inhibitor Y-27632 and MLCK inhibitor wortmannin to elucidate the potential role of PTK in the inotropic effects induced by crosstalk of ET-1 and NE in dog, and that of MLCK and Rho kinase in regulation of cardiac contractility and CaT in rabbit. Experiments were carried in isolated dog and rabbit ventricular myocardium and indo-1/AM-loaded single myocytes.

In isolated dog ventricular trabeculae and myocytes, genistein elicited a concentration-dependent PIE in association with an increase in the amplitude of CaT. Genistein potentiated the NE-induced PIE and an increase in the amplitude of CaT in a concentration-dependent mannar, but inhibited the ET-1-induced PIE and increase in the amplitude of CaT in the presence of NE at low concentrations, as well as the ET-induced NIE and decrease in amplitude of CaT in the presence of NE at high concentrations, whereas genistein did not affect the NIE of carbachol, that elicits anti-adrenergic effect via activation of G_i proteins.

In isolated rabbit papillary muscles and myocytes, Y-27632 inhibited significantly the baseline contractility and the amplitude of CaT, suppressed the ET-1-induced PIE and increase in the amplitude of CaT in a concentration-dependent manner. On the other hand, wortmannin decreased basal contractile force without affecting the amplitude of CaT, markedly inhibited the ET-1-induced PIE, but did not suppress the increase in the amplitude of CaT induced by ET-1. Neither Y-27632 nor wortmannin affected the PIE and the increase in the amplitude of CaT induced by isoproterenol (ISO) via β-adrenoceptor stimulation.

Our findings in the dog indicate that both the PIE and NIE of ET-1 in the presence of NE require the activation of PTK, and the NIE induced by ET-1 and muscarinic receptor stimulation involve different pathways in dog ventricular myocardium and myocytes. The findings in rabbit imply that the activation of ROK and MLCK contributes to the ET-1-induced increase in contractility: ROK primarily elevates increase in the amplitude of CaT, while MLCK mainly increases the myofilament Ca^{2+} sensitivity in rabbit ventricular myocardium and myocytes.

Key words: Endothelin-1, protein tyrosine kinase, Rho kinase, myosin light chain kinase, inotropic effect

緒　言：

Endothelin-1 (ET-1) is a potent vasoactive peptide of 21 amino acids that was originally isolated from the culture medium of porcine aortic endothelial cells. It exerts a positive inotropic effect (PIE) in most mammalian species but not in dog ventricular myocardium. Our recent studies in the dog heart showed that in the presence of low concentrations of β-adrenoceptor agonists, such as norepinephrine (NE) and isoproterenol (ISO), ET-1 induced a PIE, whereas it elicited a negative inotropic effect (NIE) in the presence of high concentrations of β-adrenoceptor agonists.

Protein tyrosin kinase (PTK) mediates the actions of a various types of cells including the cardiovascular systems. Recent studies revealed that the PTK inhibitor genistein enhanced an increase in L-type Ca^{2+} current ($I_{(Ca)L}$) induced by β-adrenoceptor stimulation. In contrast, however, we recently found that genistein exerted an inhibitory action on the ET-1-induced PIE and an increase in Ca^{2+} transients in rabbit ventricular myocytes, but little is known about the role of activation of PTKs in the crosstalk of ET-1 and NE in the dog heart.

The intracellular signaling pathway for cardiac regulation has been shown to involve monomeric G protein (RhoA) and its direct target Rho kinase (ROK). RhoA is activated by binding guanosine triphosphatase (GTPase), stimulates ROK, which phosphorylates and inhibits the myosin light chain (MLC) phosphatase and/or MLC itself. In smooth muscle cells, MLC kinase (MLCK) and ROK act in concert to increase phosphorylation of MLC-2. While MLC-2 is phosphorylated by MLCK also in the heart, the role of MLC-2 phosphorylation in cardiac muscle has been studied relatively less because unlike smooth muscle, cardiac muscle contraction is primarily regulated by thin filament modulation, the experiments were carrid out to elucidate whether the activation of ROK and MLCK is involved in the ET-1-induced contractility and cytosolic Ca^{2+} signaling.

In the present study, we investigated the influence of PTK inhibitor genistein to elucidate the role of PTK signaling in regulation of the inotropic effects induced by crosstalk of ET-1 and NE in isolated dog ventricular trabeculae. We also applied ROK inhibitor Y-27632 and MLCK inhibitor wortmannin to isolated rabbit papillary muscles, examined their effects on inotropic responses to ET-1. For comparison the influence of these inhibitors on the PIE mediated by β-adrenoceptor stimulation by ISO or NE was also studied. Our previous reports have indicated that the effects of ET-1 and receptor antagonists on single ventricular myocytes are quantitatively different from but qualitatively similar to those in papillary muscle. Therefore, the present experiments were also carried out in ventricular myocytes loaded with a fluorescent dye, acetoxymethylester of indo-1 (indo-1/AM).

対象と方法：

1. Preparation of dog ventricular trabeculae and rabbit papillary muscles
2. Measurements of inotropic effects in dog and rabbit ventricular myocardium.
3. Isolation of dog and rabbit cardiac ventricular myocytes.
4. Loading of myocytes with indo-1/AM.
5. Simultaneous measurements of cell shortening and indo-1 fluorescence ratio.

結　果：

1. Effects of genistein on dog ventricular trabeculae and myocytes:

(1) The PTK inhibitor genistein elicited a concentration-dependent PIE in association with an increase in the amplitude of CaT, while daidzein, an inactive analogue of genistein, elicited a NIE.

(2) Genistein but not daidzein inhibited the ET-1-induced PIE and increase in amplitude of CaT in the presence of NE at low concentrations.

(3) Genistein as well as daidzein antagonized the ET-induced NIE in association with a decrease in amplitude of CaT in the presence of NE at high concentrations, but genistein did not affect the anti-adrenergic effect of carbachol.

(4) Genistein but not daidzein enhanced the PIE in association with an increase in CaT induced by NE via the activation of β-adrenoceptors, which was abolished by the protein tyrosine phosphatase inhibitor vanadate.

2. Effects of Y-27632 and wortmannin on rabbit papillary muscles and ventricular myocytes:

(1) Y-27632 (3-30 μM) inhibited significantly the baseline contractility and the amplitude of CaT, but wortmannin decreased the basal contractile force without affecting the amplitude of CaT.

(2) Y-27632 suppressed the ET-1-induced increases in contractility and the amplitude of CaT in a concentration-dependent manner, but wortmannin only inhibited the ET-1-induced PIE, with no influence on the ET-1-induced changes in the amplitude of CaT.

(3) Y-27632 and wortmannin did not affect the effect of ISO elicited by β-adrenoceptor stimulation.

考　察：

The important findings in the present study are that the PTK inhibitor genistein elicited the action as the PTK inhibitor and non-specification that is unrelated to PTK inhibition. The action as the PTK inhibitor involves 1) an inhibition of the PIE and Ca^{2+} signal induced by crosstalk of ET-1 and NE; 2) an enhancement of the PIE and CaT induced by NE via activation of β-adrenoceptors; and 3) a direct facilitatory action on basal contractility and Ca^{2+} transients. In addition, genistein inhibited the NIE of ET-1 in the presence of high concentrations of NE, which was mimicked by an inactive analog, daidzein.

In rabbit ventricular myocardium and myocytes, ROK inhibitor Y-27632 decreased the basal force of contraction and Ca^{2+} transients, inhibits the ET-1-induced PIE and increase in Ca^{2+} transients in a concentration-dependent manner. MLCK inhibitor wortmannin at concentrations of μM decreased the basal force of contraction and inhibited the ET-1 induced PIE and the increase in cell shortening, but did not affect the amplitude of CaT, suggesting that MLCK may be involved in the increase in myofilament Ca^{2+} sensitivity. The concentration of wortmannin applied in the current study was sufficient to inhibit MLCK activity, as shown in smooth muscle. Although the potential role of PI3 kinase in the effect of wortmannnin could not be completely excluded, the observations that wortmannin up to 0.3 μM did not affect the basal force and the ET-1-induced response support the view that wortmannin primarily acted as a specific inhibitor of MLCK in current experimental conditions. Neither Y-27632 nor wortmannin affected the PIE of ISO.

These observations suggest that the activities of PTK ROK and MLCK pathways may play crucial roles in cardiac function by modulation of the basal as well as receptor-mediated control of cardiac contractility and Ca^{2+} signaling under physiological and pathophysiological conditions. The activations of PTK, ROK and MLCK play differential roles in the ET- and β-adrenoceptor-mediated positive inotropic and increase in Ca^{2+}

transients in dog and rabbit ventricular myocardium.

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