

Possible involvement of no-synthase in inotropic effect of 4',5-dihydroxy-7-methoxyflavon in rat cardiac muscle

Abstract

The aim of the present study was to determine the inotropic effect of 4',5-dihydroxy-7-methoxyflavon in the rat myocardium. The present study demonstrates that 4',5-dihydroxy-7-methoxyflavon ($1-5\mu\text{mol}\cdot\text{L}^{-1}$) showed a positive inotropic effect on rat papillary muscles that can be explained with the increase of $[\text{cAMP}]_i$ and may depend on increase of $[\text{Ca}^{2+}]_i$. Also, another possible mechanism of the positive inotropic effect 4',5-dihydroxy-7-methoxyflavon ($5\mu\text{mol}\cdot\text{L}^{-1}$) on the rat papillary muscles can be inhibited Na^+/K^+ -ATPase via activation of reverse-function of the $\text{Na}^+/\text{Ca}^{2+}$ -exchange and, consequently, increase $[\text{Ca}^{2+}]_i$. And 4',5-dihydroxy-7-methoxyflavon ($10-50\mu\text{mol}\cdot\text{L}^{-1}$) may induce a negative inotropic effect in rat cardiac muscles and this effect at least partly be mediated by NO/cGC/cGMP/PKG pathway.

Keywords: biphasic inotropic effect, papillary muscles, nitric oxide, no/cgc/cgmp/pkg, 4',5-dihydroxy-7-methoxyflavon

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Abbreviations: RyR, ryanodine receptors; SERCA, sarcoplasmic reticulum; PLN, phospholamban; ATP, adenosinetriphosphate; AC, adenylate cyclase; cAMP, cyclic adenosine 3',5'-monophosphate; PKA, protein kinase a; cGMP, cyclic guanosine monophosphate; NO, nitric oxide

Introduction

Treatment of cardiovascular diseases includes a wide range of pharmacological drugs, and plant remedies are suitable alternatives to synthetic drugs due to their availability, non-proarrhythmic characteristics, and minimal side effects.¹ Medicinal plants distributed in the Central Asia and China provides abundant and precious medicinal resources for the Traditional Medicine in treating diseases.² Flavonoids are unique secondary metabolites are synthesized in almost all plant cells, exhibiting high biological activity and due to their properties increasingly finding wide practical application in pharmacology and medicine. A wide range of biological activities and low toxicity puts them in a row promising compounds in this respect. Therefore, in pharmaceutical industry researches find cure against many various diseases, the direction of tendency is observed towards the growth of interest to search and create medical products based on bioflavonoids.³⁻⁸ Also, nowadays, in chemical researches have extracted and identified many bioflavonoid from plants.^{7,9-12} However, their mechanisms of cardio pharmacological impact have not been studied yet. Therefore, the aim of the present study was to involvement of NO-synthase in inotropic effect of 4',5-dihydroxy-7-methoxyflavon in rat papillary muscle.

Material and methods

Animals and ethics statement

This study was carried out in the Laboratory of Pharmacology of Institute of Bioorganic Chemistry of Academy Sciences of the Republic of Uzbekistan on physically fit, adult, albino rats in both

sexes (female and male) obtained from the vivarium in the Laboratory of Pharmacology. Animals had been fed with standard food and water in the vivarium. In all experiments albino rats weighing 150-200g were used ($n=11$). During the experiments, while working with experimental animals, International principles of the Helsinki Declaration and the rules of human attitudes towards animals were completely followed.

Solvents and chemicals

All reagents, which were used in experiments, were of analytic-grade (NaCl , KCl , CaCl_2 , MgSO_4 , KH_2PO_4 , glucose, NaHCO_3), (\pm)-propranolol hydrochloride, dimethylsulfoxide (DMSO) was obtained from Sigma-Chemical (St. Louis, Missouri, USA). Ca^{2+} -channels were inhibited by $0.01-1\mu\text{mol}\cdot\text{L}^{-1}$ nifedipine hydrochloride (Sigma Aldrich; Germany).¹³ Also, β -AR was inhibited by $10\mu\text{mol}\cdot\text{L}^{-1}$ (\pm)-propranolol hydrochloride. 4',5-Dihydroxy-7-methoxyflavon was isolated from plants *Dracocephalum komarovii* Lipsky (Lamiaceae), collected from the Tashkent region of Uzbekistan and presented by PhD. Students of CAS_TWAS president fellowship Chinese Academy of Sciences (Xinjiang Technical Institute of Physics and Chemistry) Zokir Toshmatov (Figure 1).

Dracocephalum komarovii Lipsky (Labiatae) is endemic species, growing only in territory of Republic Uzbekistan. *Dracocephalum komarovii* Lipsky- a perennial semishrub that grows in the vicinity of ~2300-3600 m above sea level in the western Tien Shan mountain system. This is called «buzbosh» in Uzbekistan, and the local people use the aerial parts in a tea to treat various diseases such as inflammatory diseases and hypertension.

4',5-dihydroxy-7-methoxyflavon were dissolved in dimethyl sulfoxide (DMSO<0.1% in the bath). L-NAME (N^G -nitro-L-arginine methyl ester, Sigma-Aldrich, Germany), (\pm)-propranolol hydrochloride and nifedipine hydrochloride were all dissolved in distilled water. In the experiments, modified the physiological

Krebs-Henseleit solution containing (in mM): 118 – NaCl; 4.7 – KCl; 2.5 – CaCl₂; 1.2 – MgSO₄; 1.1 – KH₂PO₄; 5.5 – glucose and 25 – NaHCO₃; pH 7.4 were used. This Krebs-Henseleit solution which was continuously bubbled with 95% O₂ and 5% CO₂ and kept

at a temperature of +36±0.5°C by means of water heating system controlled by temperature controller U1 (Russia), and flowed in and out of the organ bath at a rate of 3–5ml/min with the peristaltic pump LKB Bromma (Sweden).

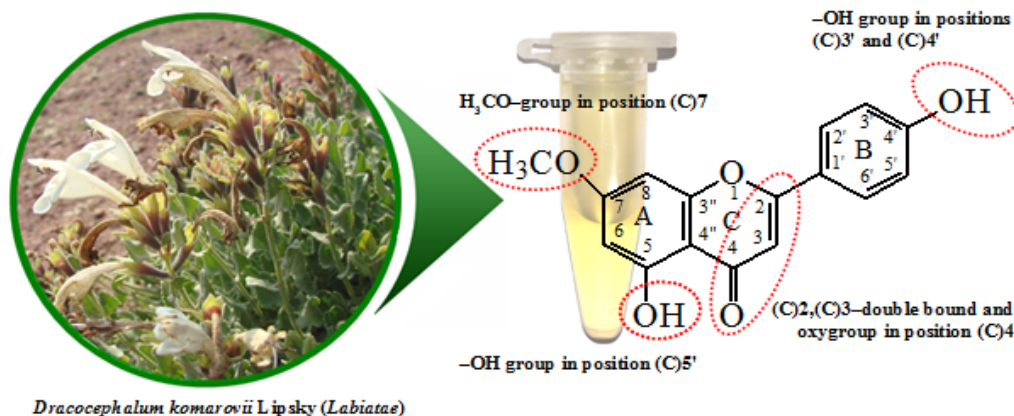


Figure 1 Chemical structure of 4',5-dihydroxy-7-methoxyflavon (C₁₆H₁₄O₉; Fw. 284g/mol).

Preparation of tissue and measurement of contractility and setup of the equipment

In experiments the papillary muscles preparations, isolated from the right atrium of adult albino rats' hearts. Rats were deeply anaesthetized with diethyl prior to paralyzing by using cervical dislocation method. The papillary muscles were ~0.4–1.3mm in diameter and 2.5–3.8mm in length. Isometric tension forces were recorded using a force

transducer (SI-KG20, World Precision Instruments, Inc. 175 Sarasota Center Boulevard, Florida 34240–9258, USA), designed for the *in vitro* study in standard pharmacological experiments for measuring contraction force response of isolated muscle preparations. The organ chamber (20ml) was part of the experimental setup, as shown in Figure 2. For further details of setup of the recording system is given in the text.

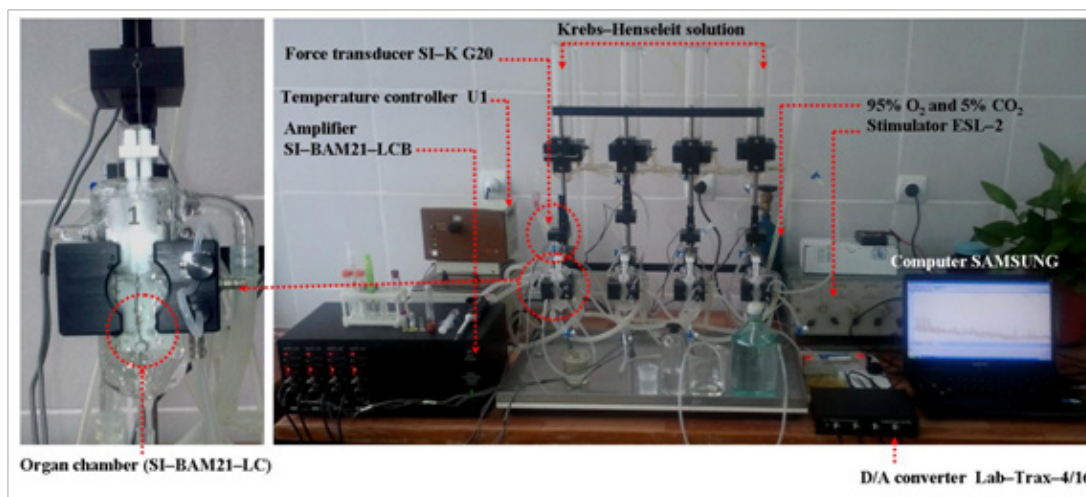


Figure 2 Schematic drawing of the experimental setup of the recording system for contractile activity of the isolated papillary muscle.

The Isometric force transducer SI-KG20 is connected to a transducer amplifier (SI-BAM21-LCB, WPI, Inc. 175 Sarasota Center Boulevard, Florida 34240–9258, USA). The papillary muscle was lifted with electric impulses that was higher than threshold (~20%), rectangular, electrical pulses of frequency 0.5Hz; 5–10msec and 5V amplitude, delivered via a pair of platinum electrodes placed in the muscle-mounting organ chamber by using stimulator ESL-2 (Russia).

Thus, wires of a pair of platinum electrodes were placed as parallel to the organ; the physiological solution of Krebs-Henseleit provides shortening the electrical contact distance between the electrodes and

the prepare of the papillary muscles. In this experiment, 10mN (1g~9.8mN) was accepted for a resting tension of the preparation papillary muscles.

After a 45–60minutes equilibration period, the length that provides development of the maximal isometric contractile force (L_{max} , the maximal length) length of the papillary muscle was found, and all experiments were carried out in these condition. After the equilibration period in the organ chamber, papillary muscles were stimulated by an initial electrical pulse of frequency 0.5Hz, amplitude 5V, and 5msec pulses. The signals obtained were given from the transducer SI-KG20

to amplifier and sent to a computer with analogue-digital converter Lab-Trax-4/16 (WPI, USA) software (iWorx LabScribe2; iWorx Systems, Inc., USA).

Data analysis

Papillary muscle contractions were plotted as a percentage of the force before the drug application in each muscle. Data were analyzed by Origin Lab Origin Pro v. 8.5 SR1 (EULA, Northampton, MA 01060-4401, USA). Pooled data are given as means \pm S.E.M. of observations (n). Concentration-response curves were fitted to the logistic equation:

$$E = E_{max} / (1 + 10^{-k \times ([drug] - pD_2)})$$

where E_{max} – is the maximal effect, k – is a factor which represents the slope of the curve, and pD_2 – is the drug concentration exhibiting 50% of the E_{max} expressed as negative log molar. Values are expressed as mean \pm S.E.M. Statistical differences of the data were calculated by ANOVA and the paired or unpaired Student's t -test where appropriate. The values were considered significantly different when $p < 0.01$ and $p < 0.05$.

Result and discussion

The positive inotropic effects of 4',5-dihydroxy-7-methoxyflavon on rat papillary muscle

In the experiments, the 4',5-dihydroxy-7-methoxyflavon (1–50 $\mu\text{mol L}^{-1}$) showed dose-dependent biphasic inotropic effects in rat papillary muscle contractility.

The condition where the maximal effected concentration (5 $\mu\text{mol L}^{-1}$) of 4',5-dihydroxy-7-methoxyflavon, the isometric developed force of papillary muscle preparation was increased from 2.6 \pm 0.1 mN (the control basal value) to 3.8 \pm 0.1 mN or 46.2 \pm 4.78% in comparison with the control group ($p < 0.05$; $n = 3-5$).

Studies have shown that in incubation to inhibited potential-dependent Ca^{2+} -channel – nifedipine hydrochloride (0.01 $\mu\text{mol L}^{-1}$) the positive effect of 4',5-dihydroxy-7-methoxyflavon (5 $\mu\text{mol L}^{-1}$) decreases to 31.5 \pm 4.7% of control values. The results of experiment demonstrate that the positive inotropic effect of 4',5-dihydroxy-7-methoxyflavon on papillary muscle is not completely connected with modulation of potential-dependent Ca^{2+} -channel cardiomyocytes. In addition, the positive inotropic effect of 4',5-dihydroxy-7-methoxyflavon (5 $\mu\text{mol L}^{-1}$) was almost completely disappeared in the presence of inhibitor of potential-dependent Ca^{2+} -channel – nifedipine hydrochloride (0.01 $\mu\text{mol L}^{-1}$) and inhibitor β -adrenoreceptor-(\pm)-propranolol hydrochloride (10 $\mu\text{mol L}^{-1}$) under incubation conditions.

Other investigations showed that flavonoids demonstrate a positive inotropic effect, through an increase in cAMP, which increases $[\text{Ca}^{2+}]_i$. It is known that, increasing concentration of $[\text{cAMP}]_i$ activates protein kinase A (PKA), which phosphorylates the L-type Ca^{2+} -channel, troponin I, and causes an increase of $[\text{cAMP}]_i$ and subsequently phosphorylation of contraction-controlling proteins, including Ca^{2+} -channels occurred, and the amplitude force of contraction papillary muscles increased as well. Phosphorylation of these Ca^{2+} -channels promotes Ca^{2+} influx that triggers the release of Ca^{2+} from the RyRs of SR and $[\text{Ca}^{2+}]_i$ transient finally activates the contraction system of myocardium.

The present study demonstrates that 4',5-dihydroxy-3,6,7-

trimethoxyflavon has showed a positive inotropic effect on rat papillary muscles that can be explained with the increase of $[\text{cAMP}]_i$ and may depend on increase of $[\text{Ca}^{2+}]_i$.

Another possible source of $[\text{Ca}^{2+}]_i$ could be found in activation of reverse-function of the $\text{Na}^+/\text{Ca}^{2+}$ -exchange.¹⁴ Also, cardiac glycosides (ouabain, digoxin et al.) exert a positive inotropic effect on papillary muscle through a mechanism the inhibition of the Na^+/K^+ -ATPase (sodium pump). This leads to an increased $[\text{Na}^+]_i$ which in turn is thought to influence the $\text{Na}^+/\text{Ca}^{2+}$ -exchange in such a way as to increase the $[\text{Ca}^{2+}]_i$ and hence the force of contraction papillary muscle.¹⁵⁻²¹ And, inhibition of Na^+/K^+ -ATPase is involved in probably to a large extent in therapeutic efficacy of cardiac glycosides.^{20,22,23}

The most frequently cardiac glycoside used in experimental research and in clinical conditions today is digoxin.²⁴⁻²⁸ Digoxin, a cardiac glycoside and digitalis alkaloid obtained from the leaves of plants of the plant *Digitalis lanata*, and used clinically in the treatment of heart failure and arrhythmias by inhibiting the Na^+/K^+ -ATPase by binding to the extracellular domain of the α -subunit of Na^+/K^+ -ATPase on cardiomyocytes. And, reported²⁹ that digoxin at 0.4 $\mu\text{mol L}^{-1}$ demonstrated a near maximal positive inotropic effect ($EC_{50} \sim 0.2 \mu\text{mol L}^{-1}$).

Given that to examine this possibility, the Na^+/K^+ -ATPase was modified by incubating papillary muscle with 1 $\mu\text{mol L}^{-1}$ digoxin. Thus, papillary muscle from rat displayed an increase of force to 87.4 \pm 6.7% of control values after digoxin (1 $\mu\text{mol L}^{-1}$; 0.5 Hz) ($n = 3-4$; $p < 0.05$). A combination of 1 $\mu\text{mol L}^{-1}$ digoxin and 4',5-dihydroxy-7-methoxyflavon (5 $\mu\text{mol L}^{-1}$) resulted in a potentiation of the developed contractile tension of the rat papillary muscles from to 15.8 \pm 4.9% of control values.

Also, reported³⁰ that flavonoids demonstrated a positive inotropic effect, through an inhibited Na^+/K^+ -ATPase *in vitro*.

These results indicate that another possible mechanism of the positive inotropic effect 4',5-dihydroxy-7-methoxyflavon (5 $\mu\text{mol L}^{-1}$) on the rat papillary muscles can be inhibited Na^+/K^+ -ATPase via activation of reverse-function of the $\text{Na}^+/\text{Ca}^{2+}$ -exchange via increase $[\text{Ca}^{2+}]_i$.

The negative inotropic effects of 4',5-dihydroxy-7-methoxyflavon on rat papillary muscle

In the experiments, the 4',5-dihydroxy-7-methoxyflavon (15–50 $\mu\text{mol L}^{-1}$) induced a concentration-dependent decrease of the contractile force of the rat papillary muscle ($p < 0.05$). The condition where the maximal effected concentration (50 $\mu\text{mol L}^{-1}$) of 4',5-dihydroxy-7-methoxyflavon, the isometric developed force of papillary muscle preparation was decreased from 54.8 \pm 6.2% in comparison with the control group ($p < 0.05$; $n = 3-4$) (Figure 3).

Stimulation: 0.5 Hz with pulses of amplitude 5V, and 5 msec (+36 \pm 0.5 $^{\circ}\text{C}$).

In these conditions, the EC_{50} value (the values of concentration for 50% of the maximal effect) 4',5-dihydroxy-7-methoxyflavon was 38.9 $\mu\text{mol L}^{-1}$ or pD_2 ($-\log EC_{50}$) = 4.415 (Figure 4).

Results are given as means \pm S.E.M, $n = 3-4$. Stimulation: 0.5 Hz, 5V, 5 msec, +36 \pm 0.5 $^{\circ}\text{C}$, resting tension = 10 mN.

In the experiments, the NO donor sodium nitroprusside (SNP) (100 $\mu\text{mol L}^{-1}$) showed a negative inotropic effect (at 0.5 Hz; $p < 0.05$).

Treatment with sodium nitroprusside (SNP) ($100\mu\text{mol}\cdot\text{L}^{-1}$) decreased maximal force by $56.4\pm 4.6\%$ compared to control conditions. These results are consistent with studies other authors.³¹ This effect is nearly eliminated by the inhibition of NO-synthases through L-NAME ($100\mu\text{mol}\cdot\text{L}^{-1}$).

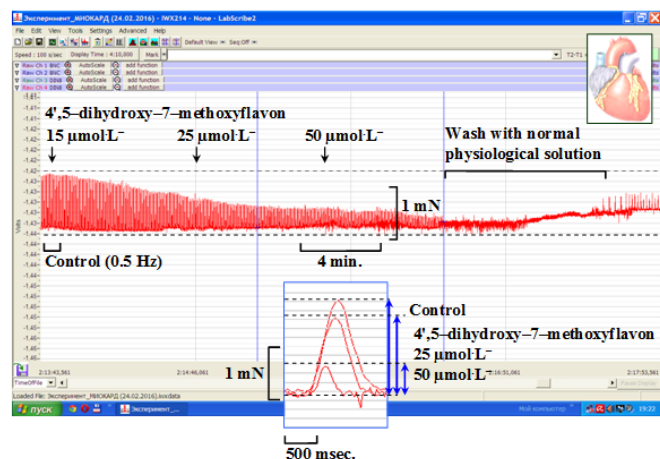


Figure 3 Original recordings of negative inotropic effect of 4',5-dihydroxy-7-methoxyflavon ($15\text{--}50\mu\text{mol}\cdot\text{L}^{-1}$) on a rat papillary muscle.

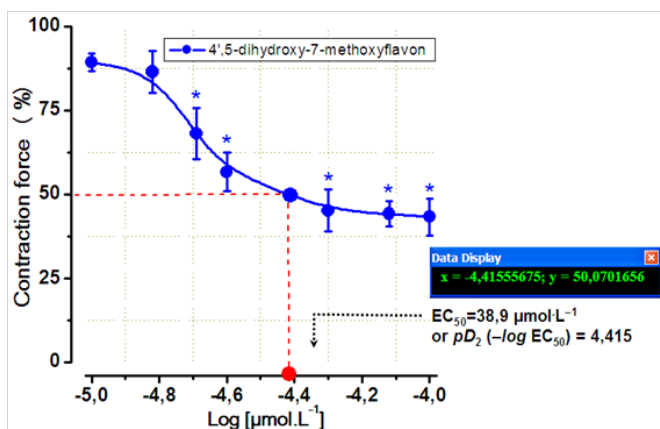


Figure 4 Concentration-response curves for the negative inotropic effects of 4',5-dihydroxy-7-methoxyflavon.

Pretreatment with L-NAME ($100\mu\text{mol}\cdot\text{L}^{-1}$), an inhibitor of NO-synthase, attenuated the negative inotropic effect of 4',5-dihydroxy-7-methoxyflavon in the isolated rat papillary muscles ($p<0.05$) (Figure 5).

Stimulation: 0.5Hz , 5V , 5msec , $+36\pm 0.5^\circ\text{C}$, resting tension= 10mN . Data shown for L-NAME ($100\mu\text{mol}\cdot\text{L}^{-1}$) and 4',5-dihydroxy-7-methoxyflavon ($50\mu\text{mol}\cdot\text{L}^{-1}$) were presented as means \pm of 3-4 experiments. $p<0.01$ and $p<0.05$ indicates value compared to control ($n=3\text{--}4$).

And, the negative inotropic effect of 4',5-dihydroxy-7-methoxyflavon ($10\text{--}50\mu\text{mol}\cdot\text{L}^{-1}$) in isometrically contracting papillary muscles seems to be mediated by NO/cGMP/PKG-cycle.

Cardio-pharmacological experimental studies in conditions *in vitro* and traditional medical literature point to the cardiovascular effects of bioflavonoids in many instances. All results indicated the potential inotropic and anti-arrhythmic effects of flavonoids in treating cardiovascular diseases. Thus, reported, that the bioflavonoids have a

positive inotropic effect, through an increase $[\text{cAMP}]_{in}$, with inhibited of phosphodiesterase ($3'\text{--}5'\text{--cAMP--phosphodiesterase}$) enzyme, also with activation of $\beta\text{-AR}$, consequently, increase $[\text{Ca}^{2+}]_{in}$.³²⁻³⁷

And, some bioflavonoids have an inhibitory effect on the $\text{Na}^+/\text{K}^+\text{-ATPase}$ $\text{Na}^+/\text{K}^+\text{-ATPase}$ via activation of reverse-function of the $\text{Na}^+/\text{Ca}^{2+}\text{-exchange}$ and, which is increase $[\text{Ca}^{2+}]_{in}$.^{37,38,39}

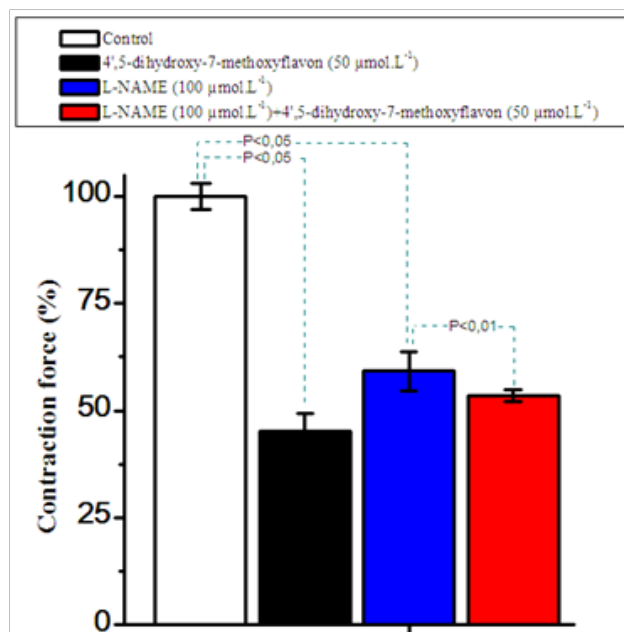


Figure 5 Comparison of the inotropic effects of 4',5-dihydroxy-7-methoxyflavon and L-NAME ($100\mu\text{mol}\cdot\text{L}^{-1}$) on the contraction force of extracted rat papillary muscle.

Conclusion

In conclusion, the present study demonstrates that 4',5-dihydroxy-7-methoxyflavon has showed a positive inotropic effect on rat papillary muscles that can be explained with the increase of $[\text{cAMP}]_{in}$ and may depend on increase of $[\text{Ca}^{2+}]_{in}$. Also, another possible mechanism of the positive inotropic effect 4',5-dihydroxy-7-methoxyflavon ($5\mu\text{mol}\cdot\text{L}^{-1}$) on the rat papillary muscles can be inhibited $\text{Na}^+/\text{K}^+\text{-ATPase}$ via activation of reverse-function of the $\text{Na}^+/\text{Ca}^{2+}\text{-exchange}$ which increase $[\text{Ca}^{2+}]_{in}$. And 4',5-dihydroxy-7-methoxyflavon ($10\text{--}50\mu\text{mol}\cdot\text{L}^{-1}$) may induce a negative inotropic effect in rat cardiac muscles and this effect at least partly be mediated by NO/cGC/cGMP/PKG pathway (Figure 6).

RyR – Ryanodine Receptors or Sarcoplasmic reticulum $\text{Ca}^{2+}\text{-channels}$; SERCA_{2a} – The sarcoplasmic reticulum $\text{Ca}^{2+}\text{-ATPase}$; PLN – Phospholamban; ATP – Adenosintriphosphat; G_s – Guanine nucleotide-binding proteins; AC – Adenylate cyclase; cAMP – Cyclic Adenosine $3',5'\text{-monophosphate}$; PKA – Proteine kinase A; cGMP – Cyclic guanosine monophosphate; PKG –cGMP-dependent protein kinase. Thus, depolarization of sarcolemmal membrane by AP leads to opening of $\text{Ca}^{2+}_L\text{-channels}$, which allows entry of Ca^{2+} into cardiomyocytes. And, through Ca^{2+} -induced activation RyR₂, leads to increase in $[\text{Ca}^{2+}]_{in}$, leading to contraction of myofilaments. During relaxation, $[\text{Ca}^{2+}]_{in}$ is accumulated into SR by SERCA_{2a} and extruded by $\text{Na}^+/\text{Ca}^{2+}\text{-exchanger}$. Agonists $\beta\text{-AR}$ through G-proteins increase $[\text{cAMP}]_{in}$, which results in activation of PKA, which leads to phosphorylation of $\text{Ca}^{2+}_L\text{-channels}$, allowing increase $[\text{Ca}^{2+}]_{in}$.

in⁺ phosphorylation of PLN, increasing SERCA_{2a} activity, induce lusitropic effect.

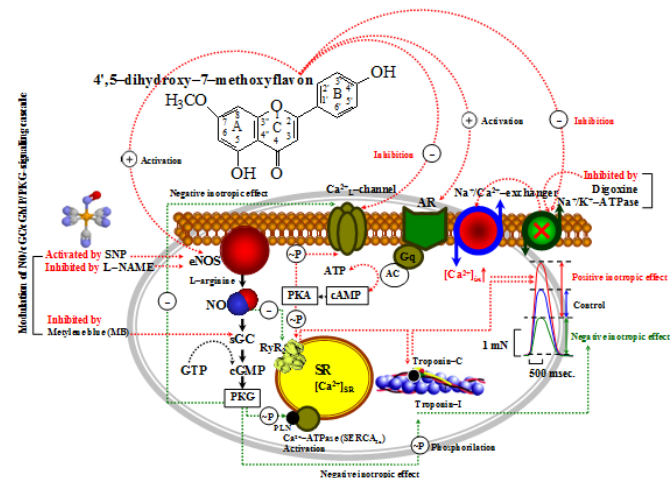


Figure 6 Hypothetical mechanisms of inotropic effect of 4',5-dihydroxy-7-methoxyflavon on the rat myocardium.

Also, phosphorylation RyR₂, induce activation release of Ca²⁺ from SR, and allowing increase [Ca²⁺]_{in}. Digoxin inhibits Na⁺/K⁺-ATPase, which increases [Na⁺]_{in}, and this results in increase in [Ca²⁺]_{in} via Na⁺/Ca²⁺-exchanger, which leads to activation SERCA_{2a} and increase in Ca²⁺ release via RyR₂.⁴⁰ NO (nitric oxide) is an important messenger that regulates physiological functions throughout the cardiovascular system,⁴¹ and NO which increase the [cGMP]_{in} level, decreased the contraction force in isolated cardiac muscle.³¹ The negative inotropic action mediated by cGC activation results from cGMP-dependent protein kinase (PKG) activation and inhibition of cAMP-phosphodiesterase (PDE III), with concomitant Ca²⁺ myofilament responsiveness reduction by troponin-I. Also, the decreased twitch tension can be due to a PKG-dependent reduction in myofilament responsiveness to Ca²⁺ in the modulation of NO.^{42,43} These data may serve as a basis for further detailed pharmacological mechanism of action of this compound.

Acknowledgements

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Conflict of interest

The author declares no conflict of interest.

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