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Effect of praeruptorin C on spontaneous $[Ca^{2+}]_i$ transients in cultured myocardial cells of neonatal rats

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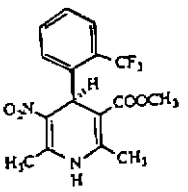
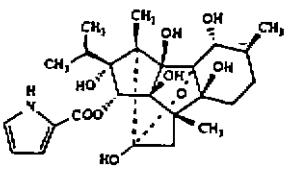
KEY WORDS coumarins; cultured cells; myocardium; calcium; Fura-2; Bay k 8644; ryanodine

AIMS: To study the effects of praeruptorin C (Pra-C) on $[Ca^{2+}]_i$ transients in cultured neonatal myocardiocytes. **METHOD**: Using Ca^{2+}-sensitive fluorescent indicator, Fura 2-AM, spontaneous cytosolic Ca^{2+} transients were measured in cultured myocardial cells of neonatal rats. **RESULTS**: Pra-C 10, 30 $\mu mol \cdot L^{-1}$ caused a decrease in the peak of Ca^{2+} transients. Pra-C 30 $\mu mol \cdot L^{-1}$ and 10 - 30 $\mu mol \cdot L^{-1}$ inhibited partly the stimulatory effects of $CaCl_2$ 4.8 $mmol \cdot L^{-1}$ and Bay k 8644 100 $nmol \cdot L^{-1}$ on peak Ca^{2+} transients, respectively. Pra-C did not cause any marked change in the basal $[Ca^{2+}]_i$ level between beats. Pra-C inhibited the reduced $[Ca^{2+}]_i$ transients after inhibition of sarcoplasmic reticulum Ca^{2+} release in ryanodine pretreated cells. **CONCLUSIONS**: Pra-C inferred with the Ca^{2+} influx responsible for excitation-contraction coupling in myocardiocytes.

Praeruptorin C (Pra-C) is a constituent of *Peucedanum praeruptorum* Dunn, the Chinese medical herb, which is used in alimentary and bronchial disorders and chest pain[1]. It inhibited the contractions induced by Ca^{2+} and K^+ depolarization in rabbit aorta[2]. It decreased the contractile effect of Ca^{2+} in K^+-depolarized swine coronary strips[3]. Pra-C had a protective effect on myocardial-reperfusion injury in rats[4]. To study mechanism of Pra-C on the heart, we investigated effects of Pra-C on $[Ca^{2+}]_i$ transients in cultured neonatal rat myocardiocytes under conditions when calcium influx, mobilization of intracellular calcium were changed by using $CaCl_2$, Bay k 8644, and ryanodine as tool agents.



Bay k 8644



Ryanodine

MATERIALS AND METHODS

Tissue culture Monolayer cultures of spontaneously contracting ventricular cells of neonatal rats were prepared[5]. Briefly, fragments of ventricles of 2 - 4 d old neonatal rats were aseptically removed and tissue fragments placed in Hanks' solution. The cells were isolated with 0.06 % trypsin at 37 ℃. To selectively enrich for myocytes, dissociated cells were preplated for 1 h, during which period the nonmyocytes (NMC) attached rapidly to the bottom of the culture dish[6]. The resultant suspension of myocytes (MC) was diluted to 1×10^9 cells$\cdot L^{-1}$ and placed in 35 mm dishes in a humidified 5 % CO_2, 95 % air atmosphere at 37 ℃. The culture medium consisted of 15 % heat-inactivated fetal serum, 75 % medium 199, 0.1 % benzylpenicillin-streptomycin solution. Bromodeoxyuridine 0.1 $mmol \cdot L^{-1}$ was added to prevent NMC proliferation without MC toxicity[7]. $[Ca^{2+}]_i$ transient was measured in myocytes at 3 d in culture.

$[Ca^{2+}]_i$ transient measurements On d 3 after dispersion, the myocytes had attached to the dishes and formed multicellular colonies of spontaneously beating cells. These spontaneously beating myocytes grown on glass coverslips were incubated with medium 199 containing Fura 2-AM 3.0 $\mu mol \cdot L^{-1}$ at 37 ℃ for 30 min and then placed in Fura-2 free medium 199 for 30 min of equilibration. The beating rate was similar to that before the addition of the Fura 2-AM.

The coverslip was placed in a cell chamber on the stage of an inverted Nikon microscope with ultraviolet epifluorescence illumination. A 40 x objective was used to view the myocytes. λ_{ex} = 340 nm/380 nm, λ_{em} = 510 nm. Photo counting data were collected from an area generally containing 5 - 10 myocytes. Maximal fluorescence for each coverslip was obtained after addition of the Ca^{2+} ionophore, ionomycin 20 $\mu mol \cdot L^{-1}$. Egtazic acid (EGTA) was added to a final

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concentration of 20 mmol · L^{-1} to obtain minimal fluorescence. Cytosolic Ca^{2+} was calculated by the following formula[8]: $[Ca^{2+}]_i = K_d \cdot (Sf_2/Sb_2) \cdot (R_{340/380} - R_{min})/(R_{max} - R_{340/380})$. K_d is the dissociation constant of Fura-2 for Ca^{2+} and was assumed to be 225 nmol · L^{-1} at 37 ℃. $R_{340/380}$ is the radio of corrected fluorescene signals. R_{max} is the radio obtained after ionomycin treatment R_{min} is the ratio of the corrected signals obtained after EGTA treatment. Sf_2 and Sb_2 represent the emission intensities at 380 nm excitation at saturating and calcium-free conditions, respectively.

Myocytes were superfused in a HEPES buffered salt solution of the following composition in mmol · L^{-1}: NaCl 135.7; KCl 5.4; KH_2PO_4 0.44; Na_2HPO_4 0.34; $NaHCO_3$ 2.62; $CaCl_2$ 1.3; $MgSO_4$ 0.81; glucose 5.6; HEPES 10 at pH 7.35. The optimal time required for stabiliation of the $[Ca^{2+}]_i$ transients after each drug was 10 min.

Fura 2-AM and ionomycin were bought from Sigma. Pra-C (light yellow power, purity >98 %) was obtained from Jiaogsu Chinese Medical Institute. Pra-C was dissolved in polyethylene glycol 400 at a concentration of 1 mmol · L^{-1}. This polyethylene glycol concentration by itself had no measurable effect. Bay k 8644 was gifted from Bayer Co, Germany.

RESULTS

$[Ca^{2+}]_i$ transients in cultured myocardiocytes Cultured neonatal rat ventricular myocytes perfused with a $[Ca^{2+}]_o$ of 1.3 mmol · L^{-1} (control) demonstrated spontaneous, regular beating activity and regular $[Ca^{2+}]_i$ transients. Each beat was accompanied by an increase in 340 nm and a decrease in 380 nm wavelength fluorescence intensity signal. Fig 1 shows a typical trace of $[Ca^{2+}]_i$ transients. The calculated maximal and minimal levels of $[Ca^{2+}]_i$ transients were 346 ± 36 and 102 ± 18 nmol · L^{-1}.

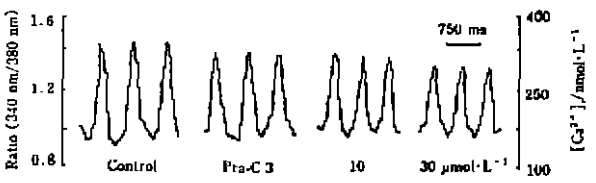

Fig 1. Effect of Pra-C on spontaneous $[Ca^{2+}]_i$ transients in cultured neonatal rat heart cells. A representative tracing of 6 experiments.

Pra-C on the $[Ca^{2+}]_i$ transients Pra-C 10 – 30 μmol · L^{-1} decreased $[Ca^{2+}]_i$ transients in a concentration-dependent fashion, but had no effect on the basal (diastolic) level of $[Ca^{2+}]_i$ and frequency of $[Ca^{2+}]_i$ transients (Fig 1, Tab 1). Pra-C 10, 30 μmol · L^{-1} inhibited the maximal levels of $[Ca^{2+}]_i$ transients by 11 % and 18 %, respectively. The effect of Pra-C on $[Ca^{2+}]_i$ transients disappeared after washout of Pra-C.

Tab 1. Effects of Pra-C on spontaneous $[Ca^{2+}]_i$ transients in cultured neonatal rat heart cells. $n = 6$, $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^dP < 0.05$, $^eP < 0.01$ *vs* $CaCl_2$. $^fP > 0.05$, $^gP < 0.01$ *vs* Bay k 8644. $^jP > 0.05$, $^kP < 0.05$ *vs* ryanodine.

Pra-C/μmol·L^{-1}	Maximal transients /nmol·L^{-1}	Frequency /times ·min^{-1}
Control	357 ± 22	82 ± 12
Pra-C (3)	356 ± 30[a]	80 ± 13[a]
(10)	319 ± 20[c]	79 ± 14[a]
(30)	293 ± 20[c]	79 ± 12[a]
$CaCl_2$(4.8) mmol·L^{-1}	503 ± 48[c]	123 ± 18[c]
Pra-C (10) + $CaCl_2$(4.8) mmol·L^{-1}	468 ± 42[e]	108 ± 12[j]
Pra-C (30) + $CaCl_2$(4.8) mmol·L^{-1}	427 ± 36[f]	96 ± 14[j]
Bay k 8644 (100) nmol·L^{-1}	453 ± 40[c]	80 ± 14[a]
Pra-C (10) + Bay k 8644 (100) nmol·L^{-1}	391 ± 37[f]	77 ± 15[g]
Pra-C (30) + Bay k 8644 (100) nmol·L^{-1}	373 ± 30[f]	76 ± 13[g]
Ryanodine (3) μmol·L^{-1}	154 ± 21[c]	78 ± 15[a]
Ryanodine (3) μmol·L^{-1} + Pra-C (30)	125 ± 18[k]	75 ± 13[j]

Pra-C on $CaCl_2$-induced $[Ca^{2+}]_i$ transients At $[Ca^{2+}]_o$ 1.3 mmol · L^{-1}, the maximal and minimal levels of $[Ca^{2+}]_i$ transients were 357 ± 22 and 82 ± 12 nmol · L^{-1}, respectively. Elevation of $[Ca^{2+}]_o$ from 1.3 to 4.8 mmol · L^{-1} was associated with an increase of both the maximal and minimal levels of the $[Ca^{2+}]_i$ transients by 41 % and 18 %, respectively. $[Ca^{2+}]_o$ 4.8 mmol · L^{-1} increased the frequency of $[Ca^{2+}]_i$ transients (times · min^{-1}) from 82 ± 12 to 123 ± 18. Preincubation with Pra-C 30 μmol · L^{-1} for 10 min, markedly inhibited $CaCl_2$-induced maximal levels of and frequency of $[Ca^{2+}]_i$ transients, but had no effect on the minimal levels of $[Ca^{2+}]_i$ transients (Tab 1).

Pra-C on Bay k 8644-induced $[Ca^{2+}]_i$ transi-

ents Tab I shows that Bay k 8644 100 nmol·L^{-1} increased the maximal levels of $[Ca^{2+}]_i$ transientss by 27 %. Preincubated with Pra-C 10 and 30 μmol·L^{-1} for 10 min, markedly inhibited Bay k 8644-induced maximal levels of $[Ca^{2+}]_i$ transients by 65 % and 83 %, respectively. Bay k 8644 and Pra-C had no effect on the minimal levels of $[Ca^{2+}]_i$ transiens and the frequency of $[Ca^{2+}]_i$ transients.

Pra-C on ryanodine-reduced $[Ca^{2+}]_i$ transients Preincubation of the cells with ryanodine 3 μmol·L^{-1} for 10 min reduced the maximal levels of $[Ca^{2+}]_i$ transients to 57 %. Pra-C 30 μmol·L^{-1} caused further reductions (19 %) in the maximal levels of $[Ca^{2+}]_i$ transients after pretreatment with ryanodine (Tab I).

DISCUSSION

In this experiment, we used Ca^{2+}-sensitive fluorescent indicator, Fura 2-AM to measure the $[Ca^{2+}]_i$ transients in cultured myocardial cells of neonatal rats. These values are comparable to the $[Ca^{2+}]_i$ transients reported in cultured cardiac cells[9].

The previous studies indicated that Pra-C inhibited markedly KCl-induced contractions of coronary artery[3], aorta, and atria[2], suggested that Pra-C had a negative inotopic effect and could block the potential sensitive channels (PSC) and decreased the Ca^{2+} influx.

In present experiments, $CaCl_2$-induced $[Ca^{2+}]_i$ transients could be attributed to increased sarcolemmal Ca^{2+} influx and subsequent increased intracellular Ca^{2+} concentrations. The increase of cytosolic Ca^{2+} during the contractile cycle of the heart cell is derived from two sources[10,11], the extracellular medium and the sarcoplasmic reticulum. There are two possible sites at which Pra-C might act to reduce $[Ca^{2+}]_i$ transients, including inhibition of sarcolemmal Ca^{2+} influx via voltage-sensitive Ca^{2+} channels and alterations of Ca^{2+} release and sequestration by the sarcoplasmic reticulum.

The dihydropyridine derivative Bay k 8644 was described as a calcium agonist, which acts directly on the voltage-sensitive Ca^{2+} channel[12]. Pra-C reduced the magnitude of Bay k 8644-induced $[Ca^{2+}]_i$ transients, indicated that Pra-C may inhibit voltage-sensitive Ca^{2+} channel. Ryanodine, a plant alkaloid, was known to deplete Ca^{2+} from SR by accelerating Ca^{2+} loss from SR as indicated by an reduction in magnitude of Ca^{2+} transient[13]. Under these conditions $[Ca^{2+}]_i$ transients depend mainly on trans sarcolemmal Ca^{2+} influx[14,15]. Pra-C could still inhibit the reduced Ca^{2+} transients in ryanodine pretreated cells in this experiment. The result support the conclusion that Pra-C acts on trans sarcolemmal Ca^{2+} flux.

The sarcoplasmic reticulum plays an important role in $[Ca^{2+}]_i$ transients of myocardial cells[10]. The effect of Pra-C on Ca^{2+} release from the sarcoplasmic reticulum requires further exploration. To our knowledge, nothing is known about effects of Pra-C on Na^+-Ca^{2+} exchange and the sarcoplasmic reticulum Ca pool. This may be also postulated as a mechanism of Pra-C induced reduction of peak $[Ca^{2+}]_i$ transiets in cardiac myocytes.

In conclusion, Pra-C treatment of cultured myocardial cells leads to a diminution of $[Ca^{2+}]_i$ transients that links cell depolarization at the sarcolemmal to the activation of the intracellular contractile apparatus. The results suggest that the negative inotopic effect of Pra-C observed in intact cardiac muscle may result in part from inference with the Ca^{2+} influx responsible for excitation-contraction coupling in ventricular myocytes.

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前胡丙素对培养新生鼠心肌细胞自发性$[Ca^{2+}]_i$瞬间变化的影响

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关键词 香豆素类；培养的细胞；心肌；钙；Fura-2；Bay k 8644；ryanodine

目的：研究前胡丙素(Pra-C)的心脏负性肌力作用的机制. **方法**：使用_Ca^{2+}敏感的荧光指示剂 Fura 2-AM，在培养的新生鼠心肌细胞测定细胞内Ca^{2+}的瞬间变化. **结果**：Pra-C 10、30 μmol·L^{-1}引起$[Ca^{2+}]_i$瞬间变化最大值明显减少. Pra-C 10，30 μmol·L^{-1}能部分抑制 $CaCl_2$ 4.8 mmol·L^{-1}及 Bay k 8644 100 nmol·L^{-1}增加的$[Ca^{2+}]_i$瞬间变化的峰值，但对$[Ca^{2+}]_i$瞬间变化的基线水平没有明显的影响. Ryanodine 3 μmol·L^{-1}使$[Ca^{2+}]_i$瞬间变化的幅度明显降低，在此基础上，Pra-C 30 μmol·L^{-1}继续抑制$[Ca^{2+}]_i$的瞬间变化. **结论**：Pra-C 在完整心肌的负性肌力作用可能与其部分抑制兴奋收缩偶联过程中的Ca^{2+}内流有关.