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Antagonistic effects of berbamine on $[Ca^{2+}]_i$ mobilization by KCl, norepinephrine, and caffeine in newborn rat cardiomyocytes[1]

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KEY WORDS myocardium; cultured cells; calcium; berbamine; verapamil; potassium chloride; norepinephrine; caffeine; fluorescent dyes; confocal microscopy

ABSTRACT

AIM: To study the effects of berbamine (Ber) on intracellular calcium concentration ($[Ca^{2+}]_i$) mobilized by KCl depolarization, norepinephrine (NE), and caffeine. **METHODS**: $[Ca^{2+}]_i$ was measured with fluorescent intensity (FI) by confocal microscope in single cultured cardiomyocytes of newborn rats loaded with Fluo 3-AM 2 $\mu mol \cdot L^{-1}$. **RESULTS**: FI value of $[Ca^{2+}]_i$ in control level was 248 ± 70 in the presence of extracellular calcium 1.5 $mmol \cdot L^{-1}$ and was not changed by Ber 3 - 30 $\mu mol \cdot L^{-1}$. KCl (60 $mmol \cdot L^{-1}$)- and NE (30 $\mu mol \cdot L^{-1}$)-induced $[Ca^{2+}]_i$ mobilizations were inhibited ($P < 0.01$) by Ber 30 $\mu mol \cdot L^{-1}$, similar to that of verapamil (Ver). The inhibitory effect of Ber on $[Ca^{2+}]_i$ induced by KCl was further increased ($P < 0.05$) in the presence of egtazic acid 3 $mmol \cdot L^{-1}$, but that on $[Ca^{2+}]_i$ induced by NE was not changed. The $[Ca^{2+}]_i$ mobilized by caffeine 80 and 160 $\mu mol \cdot L^{-1}$ in D-Hanks' solution was not affected ($P > 0.05$) by Ber and Ver. **CONCLUSION**: Ber possessed the antagonistic effects on $[Ca^{2+}]_i$ increases via voltage-dependent Ca^{2+} channel and receptor-operated Ca^{2+} channel in newborn rat cardiomyocytes, but without effect on intracellular Ca^{2+} release.

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INTRODUCTION

Berbamine (Ber), a natural dibenzylisoquinoline alkaloid derived from *Berberis vulgaris* L, showed a noncompetitive calcium antagonism like verapamil (Ver) and tetrandrine on isolated myocardial and vascular preparations in animal[1,2] and human[1,3]. Ber also showed the pharmacological effects of anti-hypertension[4], antiarrhythmia[5], and the protective effects in animal models of myocardial[6-8] and cerebral[9,10] ischemia. Ber inhibited both voltage-dependent calcium channel and receptor-operated calcium channel[11]. To further demonstrate the anti-calcium mechanisms, the effects of Ber on intracellular calcium elevated by KCl depolarization, norepinephrine (NE), and caffeine were investigated[12].

MATERIALS AND METHODS

Agents Ber crystals were provided by the Institute of Applied Ecology of Chinese Academy of Sciences and dissolved in stock solution with distilled water, pH 5.3 - 5.4 at 22 ℃. Ver (Orion Pharm) was dissolved with PBS before use. Fluo 3-AM (Molecular Probes, Eugene OR, USA) was dissolved in Me_2SO 1 $g \cdot L^{-1}$ (Sigma) and stored at - 20 ℃. Pluronic F-127, HEPES (4-(2-Hydroxyethyl)-1-piperrazineethane-sulfonic acid), caffeine, and egtazic acid (EGTA) were purchased from Sigma Co.

Preparation of cultured cardiomyocytes The hearts of 3-d Wistar rats provided by Experimental Animal Center of Harbin Medical University (Grade II, Certificate № 0921) were cut into small pieces and digested into cell suspension by 0.2 % collagenase. The single cardiomyocyte was obtained from centrifugation, resuspended in RPMI-1640 containing 20 % fetal calf serum (FCS), and reached for about

90 % after repeated purification. The cell suspension was adjusted into 5×10^8 cells · L^{-1} and plated on 25-mm round coverslip on the bottom of 6-well multidish in CO_2 incubator for 48 h.

Fluo 3-AM loading Coverslip with cultured cardiomyocytes were rinsed twice with calcium-free PBS, and loaded in Fluo 3-AM 2 μmol · L^{-1} working solution containing 0.03 % Pluronic F-127 at 37 ℃ for 40 – 60 min, and washed again with Hanks' solution (containing $CaCl_2$ 1.5 mmol · L^{-1}) to remove the extracellular Fluo 3-AM.

Measurement of $[Ca^{2+}]_i$ After Fluo 3-AM loading, the coverslip was mounted in the Auttofloul[R] cell chamber (Molecular Probes, Eugene OR, USA) with 200 or 240 μL Hanks' solution. The fluorescent intensity (FI) was detected by confocal microscope (Insight Plus-IQ, MI, Meridian, USA) and inverted microscope (IMT-II, Olympus, Japan) with 40 × objective and 488 nm blue laser for excitation and 530 nm for emission at 22 ℃. Stimulating agents 40 μL were added to the preparation between 2nd and 3rd scans. Total 24 – 30 images were scanned with each experiment and the data were stored in disk.

Statistic analysis Data were expressed as $\bar{x} \pm s$ and compared by t-test.

RESULTS

Effect of Ber on $[Ca^{2+}]_i$ induced by KCl KCl 60 mmol · L^{-1} induced $[Ca^{2+}]_i$ increase in single cultured cardiomyocyte (Fig 1).

The peak level of FI was reached at (23 ± 7) s. After the preincubation of Ber 3, 10, and 30 μmol · L^{-1} for 10 min, the control level of $[Ca^{2+}]_i$ was not changed. $[Ca^{2+}]_i$ increase induced by KCl was decreased by Ber 30 μmol · L^{-1} and Ver 10 μmol · L^{-1}. In the presence of egtazic acid 3 mmol · L^{-1}, the inhibitory effect was further increased. The time to peak was prolonged by Ber 30 μmol · L^{-1} and Ver 10 μmol · L^{-1} (Tab 1). This inhibitory effect of Ber or Ver on FI was attenuated to 522 ± 61 or 594 ± 77 ($P < 0.01$, $n = 9 - 14$) by the addition of $CaCl_2$ 30 mmol · L^{-1}.

Effect of Ber on $[Ca^{2+}]_i$ induced by NE In cultured cardiomyocyte, NE 30 μmol · L^{-1} elevated $[Ca^{2+}]_i$ concentration-dependently. $[Ca^{2+}]_i$ increase by NE 30 μmol · L^{-1} was inhibited by Ber 30 μmol · L^{-1} and Ver 10 μmol · L^{-1} ($P < 0.01$), even in the presence of EGTA 3 mmol · L^{-1}, the inhibitory effect of Ber or Ver was not changed ($P > 0.05$, Tab 1).

Tab 1. Effects of Ber 30 μmol · L^{-1}, Ver 10 μmol · L^{-1}, and egtazic acid (EGTA) 3 mmol · L^{-1} on $[Ca^{2+}]_i$ elevation by KCl 60 mmol · L^{-1} and NE 30 mmol · L^{-1} in cultured single cardiomyocytes, respectively. $[Ca^{2+}]_i$ change was represented by fluorescent intensity (FI). $n = 6 - 14$ cells from 32 newborn rats for KCl group and 5 – 17 cells from 29 newborn rats. $\bar{x} \pm s$.
$^cP < 0.01$ *vs* control. $^dP > 0.05$, $^fP < 0.01$ *vs* KCl. $^gP > 0.05$, $^iP < 0.01$ *vs* Ber. $^jP > 0.05$, $^lP < 0.01$ *vs* Ver. $^mP > 0.05$, $^oP < 0.01$ *vs* NE.

Groups	n	$[Ca^{2+}]_i$/FI Control	Peak	Time to peak/s
KCl	14	248 ± 70	760 ± 204^c	23 ± 7
Ber	14	213 ± 36	327 ± 47^f	54 ± 17^f
Ver	9	278 ± 93^d	459 ± 100^f	42 ± 11^f
EGTA + Ber	10	223 ± 46^g	211 ± 53^i	49 ± 14^g
EGTA + Ver	6	250 ± 77^j	204 ± 88^l	46 ± 15^j
NE	17	273 ± 51	842 ± 238^c	14 ± 10
Ber	13	253 ± 68^m	370 ± 91^o	39 ± 12^o
Ver	7	242 ± 38^m	314 ± 66^o	44 ± 13^o
EGTA + Ber	14	218 ± 39^g	333 ± 38^g	44 ± 9^g
EGTA + Ver	5	236 ± 69^j	342 ± 83^j	41 ± 11^j

Effect of Ber on $[Ca^{2+}]_i$ induced by caffeine In D-Hanks' solution, caffeine 80 and 160 μmol · L^{-1} increased $[Ca^{2+}]_i$ in a concentration-dependent manner, which was not influenced ($P > 0.05$) by Ber 30 μmol · L^{-1} or Ver 10 μmol · L^{-1} (Tab 2).

DISCUSSION

In the present research, the effects of Ber on $[Ca^{2+}]_i$ mobilization in cultured cardiomyocytes loaded with Fluo 3-AM were directly investigated with confocal microscope, which is the most sensitive method to detect lower fluorescence, almost no damage effect on living cell, and less light-bleaching to fluorescent probe, the combination use of Fluo 3-AM with this method made it more easy and possible to measure the change of fluorescent intensity.

The results demonstrated that Ber had no effect on

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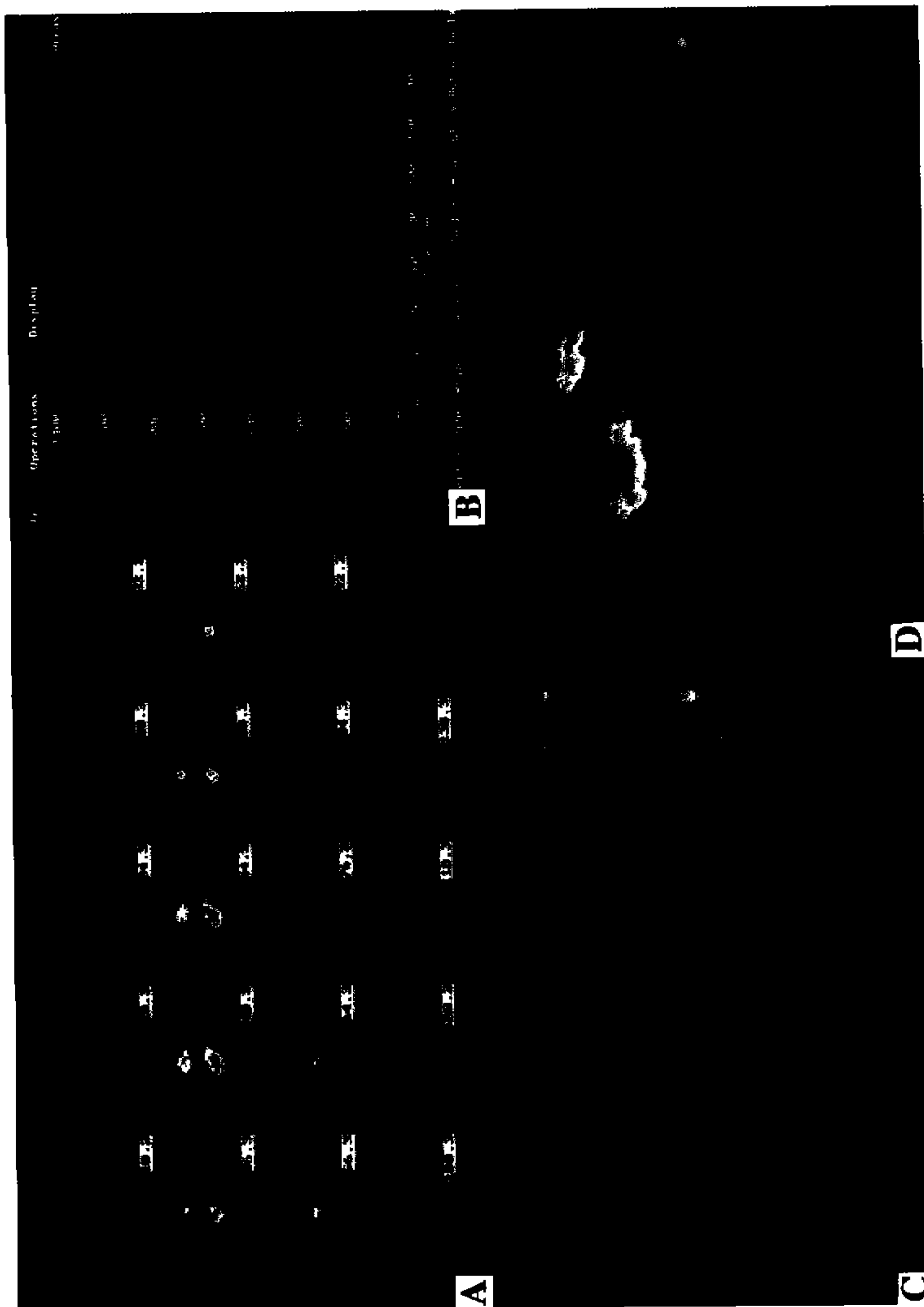

Fig 1. Fluorescent images of $[Ca^{2+}]_i$ mobilization by KCl 60 $mmol \cdot L^{-1}$ in single cultured cardiomyocyte of newborn rats loaded with Fluo 3-AM and measured by confocal microscope. A: image matrix of fluorescent images; B: the time courses of fluorescent change of cell 1 (green curve) and cell 2 (red curve); C and D: three dimensional images of control and peak states, respectively.

Tab 2. Effect of Ber 30 μmol·L^{-1} or Ver 10 μmol·L^{-1} on $[Ca^{2+}]_i$ elevation in D-Hanks' solution by caffeine in single cardiomyocyte of rats. $[Ca^{2+}]_i$ change was represented by fluorescent intensity (FI). $n = 17-21$ cells from 16 newborn rats. $\bar{x} \pm s$.
$^bP<0.05$, $^cP<0.01$ *vs* control. $^dP>0.05$ *vs* caffeine.

Groups	n	$[Ca^{2+}]_i$/FI Caffeine (μmol·L^{-1}) 0	80	160	Time to peak/s
Control	21	223 ± 37	249 ± 47^a	364 ± 45^c	15 ± 3
Ber	19	227 ± 20^d	266 ± 42^d	383 ± 93^d	13 ± 6^d
Ver	17	230 ± 37^d	254 ± 53^d	371 ± 58^d	14 ± 4^d

control level of $[Ca^{2+}]_i$, but it could inhibit the calcium influx via blocking both voltage-dependent calcium channel and receptor-operated calcium channel as well because $[Ca^{2+}]_i$ mobilizations by high K^+ depolarization and NE were significantly inhibited by Ber, and this effect of Ber on $[Ca^{2+}]_i$ was similar to that of Ver.

In addition, the results also showed that, like Ver, Ber had no effect on the intracellular calcium release from storage sites since caffeine-induced $[Ca^{2+}]_i$ elevation in D-Hanks' solution was not decreased by Ber. This action of Ber was accordant with our previous experiment[11] in which Ber only inhibited the sustained contraction caused by calcium influx in isolated basilar artery of pig by serotonin, but without the effect on the transient contraction due to calcium release. In the presence of EGTA 3 mmol·L^{-1}, the effect of Ber on $[Ca^{2+}]_i$ by KCl was enhanced and that on $[Ca^{2+}]_i$ by NE was not changed, suggesting that NE also caused the $[Ca^{2+}]_i$ release from storage sites which was not inhibited by Ber.

In conclusion, Ber inhibited the calcium influx by blocking voltage-dependent calcium channel and receptor-operated calcium channel without the effect on calcium release in cultured cardiomyocytes. Whether or not Ber can be a calcium channel blocker, calcium current recording with patch-clamp technique will be needed.

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小檗胺对高钾、去甲肾上腺素及咖啡因引起大鼠心肌细胞内钙动员的拮抗作用[1]

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关键词　心肌; 培养的细胞; 钙; 小檗胺; 维拉帕米; 氯化钾; 去甲肾上腺素; 咖啡因; 荧光染料; 共聚焦显微镜检查

目的: 研究小檗胺(Ber)对氯化钾、NE 及咖啡因引起大鼠培养心肌细胞$[Ca^{2+}]_i$动员的影响. **方法**: Fluo 3-AM 负载后, 共聚焦法测定心肌细胞$[Ca^{2+}]_i$荧光强度的变化. **结果**: Ber 对心肌细胞静息$[Ca^{2+}]_i$水平无影响, 但可剂量依赖性地抑制 KCl 60 $mmol \cdot L^{-1}$及 NE 30 $\mu mol \cdot L^{-1}$引起的内钙动员($P<0.01$), 此作用与维拉帕米相似. Egtazic acid 3 $mmol \cdot L^{-1}$并不能增强 Ber 对 NE 引起的$[Ca^{2+}]_i$升高的抑制作用. 无外钙时, 咖啡因 80 – 160 $\mu mol \cdot L^{-1}$的$[Ca^{2+}]_i$动员不受 Ber 的影响($P>0.05$). **结论**: Ber 与维拉帕米相似, 对大鼠心肌细胞靠电压依赖性和受体操纵性钙通道而升高的胞$[Ca^{2+}]_i$有拮抗作用, 并不影响$[Ca^{2+}]_i$释放.

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