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阿片、钙及肾上腺素受体与普罗托平的镇痛

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摘要 小鼠尾根部加压及热板法证实，普罗托平10—40 mg·kg^{-1}，有显著镇痛作用，抑制小鼠自发活动，促进戊巴比妥钠的催眠，但无抗炎作用。icv 20—200 μg/鼠时镇痛显著。纳洛酮、$CaCl_2$和MTX可阻断，Nif则增强其镇痛。利血平和酚妥拉明对其镇痛有抑制或抑制趋势，而普奈洛尔无影响。因此，其镇痛作用主要系阿片及钙机制，部分通过肾上腺素能机制。

关键词 普罗托平；止痛；甲氨蝶呤；硝苯地平；纳洛酮；利血平；酚妥拉明

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Effects of direct lytic factors from southern Chinese cobra venom on Ca^{2+} movement in rabbit aorta strip[1]

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ABSTRACT The purified direct lytic factors (DLF) from southern Chinese cobra (*Naja naja atra*) venom induced a contractile response in Ca^{2+}-free Krebs' solution and a further increase in the tension following a subsequent addition of Ca^{2+} into bath. After depletion of intracellular Ca^{2+} pool by phenylephrine, DLF failed to induce any contractile response. In $^{45}Ca^{2+}$ experiments, DLF increased both $^{45}Ca^{2+}$ release and $^{45}Ca^{2+}$ influx. Procaine 2 mmol·L^{-1} decreased the DLF induced $^{45}Ca^{2+}$ release and $^{45}Ca^{2+}$ influx by 67±23% and 46±32%, respectively. Nifedipine and varapamil 1 mmol·L^{-1} markedly inhibited the contractile response and the $^{45}Ca^{2+}$ influx induced by DLF. These results suggest that DLF induces extracellular Ca^{2+} entry through voltage dependent Ca^{2+} channel and Ca^{2+} release from the intracellular Ca^{2+} pool which is sensitive to phenylephrine.

KEY WORDS thoracic aorta; calcium; direct lytic factors; procaine; phenylephrine; nifedipine; verapamil

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The direct lytic factors (DLF) from cobra venom have a stimulatory effect on myocardium, skeletal muscle, vascular and other smooth muscles. These effects are suggested to be related to Ca^{2+} movement[1-3]. We found a purified DLF from crude southern Chinese cobra (*Naja naja atra*) venom which had a direct cardiotoxicity and changed cytoplasmic free Ca^{2+} concentration[4-6]. In the present study, we studied the effects of this DLF on intracellular Ca^{2+} release and extracellular Ca^{2+} entry in rabbit aorta strip.

MATERIALS AND METHODS

Preparation of aorta New Zealand rabbits of either sex weighing 1.7±0.3 kg were killed by stunning following exsanguination. Segments of thoracic aorta were cut into small strips (about 2 mm in width and 3.5 mm in length) in normal Krebs' solution (NaCl 115.0, KCl 4.6, $MgSO_4$ 1.2, $NaHCO_3$ 21.9, NaH_2PO_4 1.1, $CaCl_2$ 2.5, and glucose 11.0 mmol·L^{-1}, pH 7.4). For ^{45}Ca uptake and efflux experiments, the aorta was cut into small rings (20 — 40 mg). Vascular endothelium was removed by rubbing inner surface of vessels. The effectiveness of removal of endothelium was confirmed by the absence of endothelium-dependent relaxation in the presence of acetylcholine 1 μmol·L^{-1}, which in fact caused a slight contraction after rubbing. The strips were suspended in 4 ml organ bath containing normal Krebs' solution at 37℃ gassed with 95% O_2 + 5% CO_2 and allowed to equilibrate for at least 2 h under 3 g preload, which gave the maximal contractile response to KCl 100 mmol·L^{-1}.

Contractility The bath solution was changed every 20 min during the equilibration period. Stable contractile responses induced by KCl 100 mmol·L^{-1} were obtained prior collection of data. The contraction was considered reproducible if maximal tension of two consecutive contractions differed by <10%. The preparations that failed to produce reproducible contraction were discarded. The contractile tension were expressed as g/g wet strip.

The strip was washed with Ca^{2+}-free Krebs' solution which had the same composition as that of normal Krebs' solution except for the exclusion of calcium ion and addition of EGTA 50 μmol·L^{-1}. DLF and other agents were added to the medium after incubation for 5 min in this Ca^{2+}-free Krebs' solution. When the response reached the maximum, $CaCl_2$ 2.5 mmol·L^{-1} was added into the bath to obtain additional contraction presumably due to extracellular Ca^{2+} entry. When the responses reached a plateau, the preparations were thoroughly washed with normal Krebs' solution and given an incubation for 90 min to allow refilling of intracellular calcium storage site and restoring the contractility to DLP. The concentration of EGTA (50 μmol·L^{-1}) and the incubation time (5 min) used in this study had been carefully determined not to compromise the intracellular calcium pool[7]. Under this condition, DLF induced reproducible contractile response.

$^{45}Ca^{2+}$ efflux The preparations were equilibrated at 37℃ for 90 min in HEPES (*N*-[2-hydroxyethyl] piperazine-*N*'-[2-ethanesulfonic acid]) buffer solution (NaCl 160, KCl 4.5, $MgCl_2$ 1.0, $CaCl_2$ 1.5, glucose 10, HEPES 5 mmol·L^{-1}, pH 7.2) and then incubated in a low Ca^{2+} HEPES buffer solution ($^{45}CaCl_2$ 0.2 mmol·L^{-1}, 37 MBq·L^{-1}) for 20 min. $^{45}Ca^{2+}$ bound on the external surface of membrane was washed away by stirring the preparation in a high $CaCl_2$ HEPES buffer solution ($CaCl_2$ 20.5 mmol·L^{-1} containing EGTA 20 mmol·L^{-1}) for 6 s. Then the preparations were moved from one into another of a series of tubes at 10 min intervals containing HEPES buffer solution 2 ml at 37℃. The radioactivity in each tube and tissue determined by Beckman LS-3801 liquid scintillation counter were totaled and the results were expressed as the loss of $^{45}Ca^{2+}$ (pmol·L^{-1}·min^{-1}) in each tube[8].

$^{45}Ca^{2+}$ influx The experiment was carried out as described in our previous report[9]. Aorta ring was equilibrated at 37℃ in HEPES buffer solution for 60 min, then in HEPES buffer solution labeled with $^{45}Ca^{2+}$ (18.5 MBq·L^{-1}) for 30 min. The ring was transferred to [^{45}Ca]HEPES buffer solution containing the agents to be tested and incubated for 10 min. The ring was put into ice-cold $LaCl_3$ solution ($LaCl_3$ 75.0, HEPES 20.0, glucose 10.0 mmol·L^{-1}, pH 6.9). The ring was blotted with Whatman № 1 filter paper and weighed. The $^{45}Ca^{2+}$ taken up by ring was extracted by overnight incubation in 2 ml of EGTA 5 mmol·L^{-1} at 20—23℃. Scintillation cocktail (10 ml) was added and counted in a Beckman LS-3801 liquid

scintillation counter. $^{45}Ca^{2+}$ uptake was calculated according to the method of Jim *et al*[10].

Drug Procaine (Sigma), phenylephrine (Sigma), and verapamil (Sigma) were dissolved in saline. Nifedipine (Sigma) was dissolved (10 mmol·L^{-1}) and stored in the dark in 100% ethanol and freshly diluted with demineralized water. It was used under subdued lighting. DLF was separated and purified from venom of *Naja naja atra* (southern Chinese cobra) by ion exchange chromatography according the method of Lee *et al*[8]. Purified DLF showed a single band on both polyacrylamide gel electrophoresis and immunoeletrophoresis. The molecular weight of DLF was 7033 estimated according to its R_f value on SDS slab electrophoresis. Its IV LD_{50} on mice was determined to be 1.3 mg·kg^{-1} (95% confidence limit: 1.0—1.5 mg·kg^{-1}) with Bliss method. The DLF used in the present study was the same batch obtained in previous study[6]. $^{45}CaCl_2$ (122.8 GBq·mol^{-1}) was obtained from Institude of Atomic Energy, Chinese Academy of Sciences.

RESULTS

Contractile response in Ca^{2+}-free Krebs' solution DLF (1—10 mg·L^{-1}) evoked a concentration-dependent contractile response. DLF 10 mg·L^{-1} induced a contractile response of 6.3±4.1 g/g wet strip. Subsequent addition of $CaCl_2$ 2.5 mmol·L^{-1} induced a further increase of 11.3±3.0 g/g wet strip in tension. These contractile responses induced by DLF and $CaCl_2$ were inhibited by preincubation of the preparations with procaine 2 mmol·L^{-1}, an inhibitor of intracellular Ca^{2+} release[10], by 67±23 and 46±32%, respectively. These inhibitory effects of procaine were nearly the same as those on Ca^{2+} release and Ca^{2+} entry induced by phenylephrine 10 μmol·L^{-1}, a selective α_1-adrenoceptor agonist (decreased by 73±15% and 44±10%, respectively). After depletion of intracellular Ca^{2+} pool by phenylephrine 100 μmol·L^{-1}, DLF no longer induced contractile response in Ca^{2+}-free Krebs' solution (Fig 1).

Tab 1. Effects of procaine 2 mmol·L^{-1} on rabbit thoracic aorta strip contractile responses induced by DLF 10 mg·L^{-1} and phenylephrine (Phe) 10 μmol·L^{-1} in Ca^{2+}-free solution and subsequent addition of Ca^{2+}. $^{*}P<0.01$ vs procaine-free group.

Ca^{2+}	n	Contractile tension, g/g wet strip	
		No procaine	Procaine
DLF	− 9	6.3±4.1	1.9±2.4*
DLF	+ 9	11.3±3.0	6.1±4.5*
Phe	− 8	125±55	34±32*
Phe	+ 8	282±42	221±53*

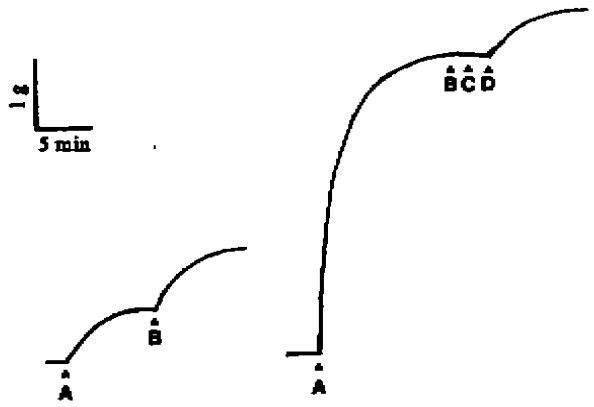

Fig 1. Rabbit thoracic aorta strip contractile responses induced by DLF and phenylephrine in Ca^{2+}-free solution. On the left, the contractile response to DLF 10 mg·L^{-1} (A) was further increased by addition of phenylephrine 10 μmol·L^{-1} (B). On the right, phenylephrine 100 μmol·L^{-1} (A) induced maximal contraction due to depletion of intracellular calcium pool, because additional phenylephrine 10 μmol·L^{-1} (B) did not cause further contraction. DLF 10 mg·L^{-1} (C) did not induce any response. But $CaCl_2$ 2.5 mmol·L^{-1} (D) caused further contraction because of Ca^{2+} influx from extracellular fluid.

Contractile response in normal Krebs' solution DLF 10 mg·L^{-1} induced contractile response of 18.6±6.3 g/g wet strip (n=58). Nifedipine and verapamil reduced the precontractile response induced by DLF 10 mg·L^{-1}. These inhibitory effects were concentration-dependent. Nifedipine 0.1, 1, and 10 μmol·L^{-1} decreased precontraction by 25±11, 39±

23, and 58±21%, respectively. Preincubation of nifedipine 1 μmol·L^{-1} (n=11) or verapamil 1 μmol·L^{-1} (n=16) for 20 min competitively blocked DLF induced contractile response.

$^{45}Ca^{2+}$ efflux and $^{45}Ca^{2+}$ influx DLF 10 mg·L^{-1} increased $^{45}Ca^{2+}$ efflux which expresses intracellular Ca^{2+} release (Tab 2). DLF 10 mg·L^{-1} increased $^{45}Ca^{2+}$ influx. The net $^{45}Ca^{2+}$ influx induced by DLF was 93±49 pmol/g wet ring (n=11). Nifedipine and verapamil 1 μmol·L^{-1} decreased the net $^{45}Ca^{2+}$ influx induced by DLF to 16±8 (n=12, $P<0.01$) and 21±13 pmol/g wet ring (n=14, $P<0.01$), respectively (Tab 3).

Tab 2. Loss of $^{45}Ca^{2+}$ (pmol·g^{-1}·min^{-1}) after DLF 10 mg·L^{-1} and phenylephrine (Phe) 1 μmol·L^{-1}. n=6. $\bar{x}\pm s$. $^{a}P>0.05$, $^{b}P<0.01$ vs control.

Time min^{-1}	Control	DLF	Phe
10	97±15	93±9^{a}	101±16^{a}
20	61±9	58±8^{a}	59±7^{a}
30	31±4	33±3^{a}	30±2^{a}
40	17±2	14±1^{a}	16±2^{a}
50	12±2	17±3^{c}	25±2^{c}
60	9±1	8±1^{a}	15±2^{c}
70	8±1	7±1^{a}	8±1^{a}
80	8±1	7±1^{a}	8±1^{a}

Tab 3. Effects of verapamil and nifedipine 1 μmol·L^{-1} on DLF (10 mg·L^{-1})-induced $^{45}Ca^{2+}$ influx (pmol/g wet ring). $^{b}P<0.01$ vs vehicle; $^{f}P<0.01$ vs DLF+vehicle.

	n	Vehicle	n	DLF
Vehicle	10	69±15	11	162±49^{b}
Verapamil	11	67±19	12	88±13^{f}
Nifedipine	12	80±14	14	96±8^{f}

DISCUSSION

The present data further confirm that DLF induces both intracellular Ca^{2+} release and extracellular Ca^{2+} entry in the vascular smooth muscle. These results are consistant with our previous results from rat lacrimal acinar cell[5]. The interesting thing was that the intracellular Ca^{2+} pool from which DLF induced Ca^{2+} release was sensitive to α_1-adrenoceptor agonist. It has been known that activation of postsynaptic α_1-adrenoceptor in vascular smooth muscle is associated with hydrolysis of phosphatidy-linositol 4,5-bisphosphate to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, then IP_3 activates the IP_3 receptor on the surface of intracellular stores resulting in Ca^{2+} release. Receptor-mediated extracellular Ca^{2+} enter may be coupled with the above event[12,13]. It has been reported that DLF cause hydrolysis of [^{3}H]inositol phospholipids and increase cytoplasmic free Ca^{2+} in rat basophilic leukemia-2H3 and Madin-Darby canine kidney cell[14]. Thus, it looks as if mechanism by which DLF induces intracellular Ca^{2+} releases is the same as that linked to activation of receptor. Our data do not provide any support for the hypothesis that intracellular Ca^{2+} release is linked to extracellular Ca^{2+} entry, because procaine decreased the responses to both DLF and phenylephrine in Ca^{2+}-free Krebs' solution by 67±23 and 73±15%, respectively and less inhibited responses to subsequent addition of $CaCl_2$(decreased by 46±32 and 44±10%, respectively). Therefore, these less inhibitions of Ca^{2+} entry by procaine may be mainly due to the direct effect of procaine on Ca^{2+} channel[15]. The present results show that DLF induced extracellular Ca^{2+} entry through voltage-dependent Ca^{2+} channel (VDC) which was sensitive to nifedipine and verapamil. Base on present results, we still could not confirm whether DLF evoked VDC opening through direct or indirect mechanisms. But we can exclude the possibility that Ca^{2+} entry through membrane pore made

by DLF at least in rabbit aorta strip, because a long incubation with DLF (10 mg·L^{-1}; this concentration was the maximum effect concentration) for 3 h, the preparation still responded with a marked contractile response to high K^+ which was sensitive to nifedipine (data not shown). The present data further support our previous suggestion that specific molecular mechanism may be involved rather than a non-specific action such as cell membrane destruction[4,6].

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眼镜蛇毒直接溶解因子对兔主动脉条 Ca^{2+} 动员的影响

R996.3

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摘要 在无 Ca^{2+} Krebs 液中，DLF 引起兔主动脉条明显收缩，加入 $CaCl_2$ 能使收缩进一步增强，这些反应可被普鲁卡因抑制。用苯福林耗竭胞内 Ca^{2+} 池后，DLF 不再引起收缩反应。DLF 引起兔主动脉环 $^{45}Ca^{2+}$ 外溢与内流。硝苯地平与维拉帕米能明显抑制DLF的 $^{45}Ca^{2+}$ 内流作用。结果提示 DLF 可能通过开放电位依赖性 Ca^{2+} 通道引起 Ca^{2+} 内流，并促进 Ca^{2+} 从苯福林敏感的胞内 Ca^{2+} 池释放出来。

关键词 胸主动脉；钙；直接溶解因子；普鲁卡因；苯福林；硝苯地平；维拉帕米